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(71) Applicants *(for all designated States except US)*: PHARMAPRINT, INC. [US/US]; Suite 1900, 4 Park Plaza, Irvine, CA 92614 (US). UNIVERSITY OF SOUTHERN CALIFORNIA [US/US]; University Park, Los Angeles, CA 90089 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KHWAJA, Tasneem, A. [US/US]; 7 Monterey Circle, Corona Del Mar, CA 92625 (US). FRIEDMAN, Elliot, P. [US/US]; 2160 Veloz, Montecito, CA 93108 (US).

(74) Agents: BALDWIN, Geraldine, F. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

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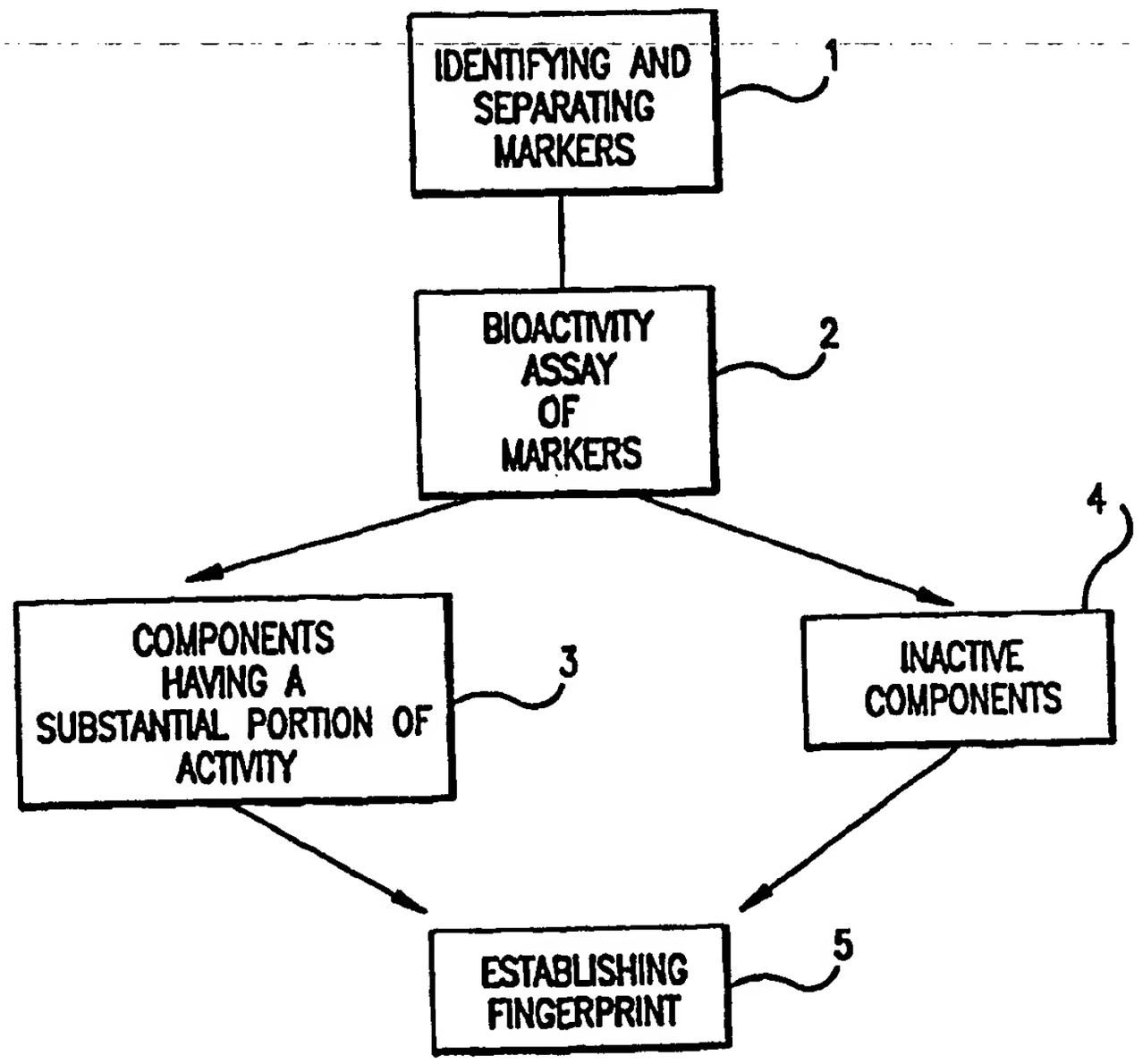
(57) Abstract

The present invention relates generally to echinacea materials and methods for making such materials in medicinally useful and pharmaceutically acceptable forms. More particularly, the present invention relates to the use of compositional and activity fingerprints in the processing of echinacea materials to produce drugs which qualify as pharmaceutical grade compositions which are suitable for use in clinical or veterinary settings to treat and/or ameliorate diseases, disorders or conditions.



PHARMACEUTICAL GRADE ECHINACEA

This is a continuation-in-part of co-pending U.S. Serial No. 08/956,603, filed on October 23, 1997, continuation-in-part of co-pending U.S. Serial No. 08/838.198, filed on April 15, 1997, the entire disclosures of which are incorporated herein by reference, which is a continuation-in-part of co-pending U.S. Serial No. 08/632,273, filed on April 15, 1996, which is a continuation-in-part of U.S. Serial No. 08/421,993, filed on April 14, 1995 abandoned in favor of U.S. Serial No. 08/774,550, filed February 4, 1997.

1. FIELD OF THE INVENTION

The present invention relates generally to botanical materials and methods for transforming such materials into medicinally useful and pharmaceutically acceptable forms. More particularly, the present invention relates to the use of compositional and activity fingerprints in the processing of a botanical material. *e.g.*, echinacea. to produce botanical drugs which qualify as pharmaceutical grade compositions which are suitable for use in clinical settings to treat and/or ameliorate diseases, disorders and/or conditions.

2. BACKGROUND OF THE INVENTION

Pharmaceutical manufacturing is based on control over the composition and bioactivity for each manufactured batch. This standardization and control provides reproducible material in the predictable and consistent treatment of patients. Herbal medicines, produced from botanical materials. have presented a unique problem for manufacturers desiring the control. reproducibility. and standardization that are required of pharmaceuticals. This problem is primarily due to the plurality of components contained in an herbal medicine and the large variation in composition and potency due to the growing, harvesting and processing conditions of raw materials.

Plants have been, and continue to be, the source of a wide variety of medicinal compounds. For centuries, various forms of botanically derived materials have been used to treat countless different ailments. The botanical materials have typically been in the form of powders made from one or more plants or plant parts or extracts derived from whole plants or selected plant parts. These powders and extracts are, for the most part, complex mixtures of both biologically active and biologically inactive compounds.

Although plant powders and extracts have been used widely for medicinal purposes, there are a number of problems associated with the use of such medicaments. For example, the complex chemical nature of the botanical materials makes it difficult to use the botanical

materials in any type of controlled and predictable manner. The potential variations in the chemical composition of different batches of material obtained from different plant harvests makes such materials unsuitable for use in clinical situations.

On a positive note, the complex groupings of bioactive components typically found in botanical materials presents the potential for synergistic or additive bioactivity profiles. However, these potential increases in medicinal effectiveness have not been predictable due to the unknown nature of these complex materials.

The above problems associated with the inherent chemical complexity of botanical medicaments has resulted in a great deal of effort being directed to the separation and isolation of the biologically active components from numerous medicinally important botanical materials. This area of endeavor has expanded rapidly in conjunction with the many improvements in chemical separation and analysis technology. Once isolated and purified. the various active components may be used in clinical settings to establish the medicinal effectiveness of a specific component. Separation and purification of individual components from botanical materials is the cornerstone of this type of drug development procedure. Once purified, the suspected active component is typically mixed with a pharmaceutically acceptable carrier and subjected to further studies in laboratory animals and eventual clinical trials in humans. Upon proof of clinical efficacy. these types of drugs are considered to be pharmaceutical grade because they contain a single, or at most a small number of, well-characterized compounds which are present in known quantities.

Pharmaceutical grade drugs are advantageous in that they allow careful tracking of the effects of individual compounds in treatment protocols. Further. the dosage of the drug can be carefully controlled to provide relatively predictable medicinal action. A disadvantage of the relative purity of such pharmaceutical grade drugs is that the potential for complex and synergistic biological activity provided by naturally occurring plant materials is reduced because of the isolation of the drug from its natural environment. The study of isolated products may also represent artifacts produced by breakdown of sensitive biological/botanical complexes. The potential benefit provided by such synergistic activity is believed by many industry experts to be outweighed by the clinical risks associated with the use of complex plant materials which are not well characterized or controlled in a clinical setting.

Although isolation and purification of single compounds from plant materials has been a popular form of drug research and development, there has also been interest in studying complex botanical extracts to characterize their medicinal qualities. Many complex plant materials and extracts exist which have potent, but relatively unpredictable,

medicinal properties. These materials are, for the most part, useless in a clinical setting because of the inherent risks involved with treating patients with poorly characterized materials which have no established batch consistency and which may differ widely in composition. Accordingly, there is a need to provide methods for standardizing such complex botanical materials so that they may be used more effectively in clinical research and patient treatments.

2.1. ECHINACEA

Echinacea refers to a genus of nine herbaceous perennial species native to North America. The term echinacea usually designates *E. angustifolia* and/or *E. purpurea*. *E. pallida* was once considered botanically synonymous with *E. angustifolia* and is often mistaken for it in commerce. *Parthenium integrifolium*, an unrelated plant has been a persistent adulterant to supplies of echinacea root (Leung and Foster, 1996, Encyclopedia of Common Natural Ingredients Used in Food, Drugs, and Cosmetics 2nd ed., New York, John Wiley and Sons, p. 216). *Echinacea angustifolia*, often called purple cornflower, is a plant of North American origin. Native Americans have used extracts from this plant for wound healing (antibiotic) and as an anti-inflammatory agent.

Echinacea was well known by the Plains Indians of North America who used the root as an antidote for snake bite and for wound healing and anti-inflammatory purposes. Traditional commercial use in the US typically employed alcoholic extracts of *E. angustifolia* root. H.C.F. Meyer brought one such preparation to the attention of the Eclectic physicians around 1870. The Eclectics then popularized and championed the use of echinacea, especially for septic conditions. It is said to have been the most widely used plant drug in the United States in the 19th century (Bisset ed., 1994, Herbal Drugs and Phytopharmaceuticals, Medpharm, Stuttgart, Germany, p. 182). *E. angustifolia* preparations were listed in the US N.F. from 1916-1950. Research in Germany by Madaus focused on the fresh pressed juice from the aerial portions of *E. purpurea* preserved with 22% ethanol.

Although there are chemical differences between different echinacea species it appears that they may be used interchangeably. Compound classes responsible for echinacea's activity appear to be primarily polysaccharides, cichoric acid and other caffeic acid derivatives, alkylamides, ketoalkynes and ketoalkenes.

The roots of echinacea are considered to form the basis of the crude drug. The aerial portion of *E. purpurea*, particularly the flower heads, is also considered medicinally useful and may be as important as the roots from the standpoint of bioactivity. An important marker of bioactivity, the alkylamides, are easily detected by the tingling sensation they produce in the mouth.

One form of echinacea usually employed is as a 1:5 tincture made with approximately 50% ethanol and taken up to teaspoonful doses several times per day. Dried powdered material as well as dried extract powders are also available. Echinacea may be administered in a dose of 900 mg per day. Echinacea is also administered in the form of tablets or capsules in a dose of 1 g three times daily. Echinacea has been approved by the German Commission E Monograph (B Anz. No. 162, dated August 29, 1992).

Freshly squeezed juices of leaves and roots echinacea have been approved by the German government for the treatment of recurrent infections of respiratory and urinary tracts. Liquid echinacea preparations have been shown to have immune stimulatory activity when administered orally or parenterally. It is believed that the activation of splenocytes may contribute to the echinacea's ability to enhance the activity of granulocytes and phagocytes.

2.2. CLINICAL INDICATIONS

The clinical indications of echinacea are many. The primary indication is increasing resistance to infection, colds, etc., in the upper respiratory tract. Other indications are superficial wound healing, treatment of recurring urinary tract infections or respiratory tract infections, herpes simplex, anti-inflammatory activity and anti-bacterial activity.

The action of echinacea appears to be that of nonspecific immune enhancement. It may act by activating macrophages. The fractions that have shown the greatest bioactivity in in-vitro tests are lipophilic fractions of *E. angustifolia*, *E. pallida* and *E. purpurea* roots, and hydrophilic fractions of *E. purpurea*. Preparations of the aerial portions of E. purpurea have been approved by the German Commission E for supportive therapy for colds and for the treatment of recurrent infections of the respiratory and urinary tracts.

The beneficial clinical effects of echinacea in treatment of infections are attributed to the ability of the drug to act as an immunostimulant. Echinacea increases phagocytosis and promotes lymphocyte activity, resulting in the release of tumor necrosis factor. Adrenal cortex activity is stimulated, and hyaluronidase activity is inhibited. Echinacea may also increase interferon production (Tyler, 1994, Herbs of Choice, Haworth Press, NY). These mechanisms tend to increase resistance to bacterial activity (Haas, 1991, Arzneipflanzenkunde, B.I. Wissenschaftsverlag, Mannheim, 134-135). Parenteral use of echinacea should not exceed three weeks; internal (non-parenteral) or external use of echinacea should not exceed eight weeks. Echinacea should not be used by persons with progressive systemic illness such as tuberculosis, leukosis, collagen diseases, multiple sclerosis, HIV infection, AIDS, or various autoimmune diseases, nor by persons who are

pregnant or with known allergy to any plant of the Asteraceae. Administration of echinacea to diabetics may worsen the condition (German Commission E Monograph, Echinaceae, August, 1992). No interactions of echinacea with other drugs have been reported.

2.2.1. PRIMARY INDICATION

The primary indication for use of echinacea is improving resistance to upper respiratory tract infections. Oral consumption of echinacea is primarily (but not exclusively) used for preventing and treating the common cold and associated conditions, such as sore throat. Preparations of fresh above ground parts of Echinacea purpurea are approved by the German Commission E as supportive therapy for treating recurring respiratory infections.

Dosage of echinacea depends upon the potency of the sample or preparation used. For hydroalcoholic preparations, 15 to 30 drops (0.75 to 1.5 mL) two to five times daily are recommended. The German Commission E recommends 8 to 9 mL daily of the juice of the overground plant portions. A decoction of 2 teaspoons of coarsely powdered herb in 1 cup (240 mL) are occasionally used, but not recommended (as not all constituents are water soluble).

2.2.2. OTHER INDICATIONS

Echinacea is also indicated as supportive therapy for recurrent urinary tract infection; topical applications of Echinacea are indicated for promotion of wound healing and skin inflammation. Other possible medical uses for echinacea include treatment of yeast infection, radiation therapy side effects, rheumatoid arthritis, blood poisoning, and food poisoning (German Commission E Monograph, Echinaceae, August, 1992; Bisset, 1994, Max Wichtl's Herbal Drugs & Phytopharmaceuticals, Boca Raton, CRC Press; Awang et al., 1991, Herbal Medicine, Echinacea. Canadian Pharmaceutical Journal, 124: 512-516; Iwu, 1993, Handbook of African Medicinal Plants, Boca Raton, CRC Press). Echinacea may fight cancer by stimulation of key lymphocyte production, which, in turn, triggers activation of natural killer cells that destroy tumor cells.

3. SUMMARY OF THE INVENTION

This invention provides a method for determining whether echinacea is a pharmaceutical grade echinacea. The method is the process of PharmaPrinting™. In one embodiment, the method comprises the steps of: providing a botanical material, such as echinacea, which comprises a plurality of components which have a given biological

activity; removing a representative aliquot from the botanical material; separating the aliquot into a plurality of marker fractions wherein each of the marker fractions comprises at least one of the active components: determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the aliquot; and comparing the bioactivity fingerprint of the aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade echinacea to provide a bioactivity fingerprint comparison to determine whether the botanical material is a pharmaceutical grade echinacea based on the bioactivity fingerprint comparison.

This invention also provides a method comprising the steps of: providing a botanical material which has a given biological activity, said botanical material comprising a plurality of components; separating a representative aliquot of the botanical material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active component; determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the representative aliquot; and comparing the bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade echinacea to determine whether the botanical material is a pharmaceutical grade echinacea.

In one embodiment, one or more of the marker fractions contain one active component.

The method may also comprise the additional steps of determining the amount of the active components in each of the marker fractions to provide a quantitative compositional fingerprint of the aliquot and comparing both the quantitative compositional and bioactivity fingerprints with a quantitative compositional and bioactivity fingerprint standard to determine whether the botanical material is a pharmaceutical grade echinacea. The method may also comprise the additional steps of: determining a total bioactivity of the aliquot of the botanical material and comparing the total bioactivity of the aliquot with that of a total bioactivity of a standard which has been established for a pharmaceutical grade echinacea.

The invention also provides a method for making a pharmaceutical grade echinacea, the method comprising the steps of: providing a botanical material which comprises a plurality of components which have a given biological activity and wherein each active component has a standardized bioactivity profile; removing a representative aliquot from the botanical material; separating the aliquot into a plurality of marker fractions wherein each of the marker fractions comprises at least one of the active components; measuring the amount of each of the active component(s) present in each of the marker fractions; calculating the

bioactivity of each of the marker fractions based on the amount of each active component present and the standardized component bioactivity profile to provide a calculated bioactivity fingerprint of the aliquot; comparing the calculated bioactivity fingerprint of the aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade echinacea to provide a bioactivity fingerprint comparison to determine whether the botanical material is a pharmaceutical grade echinacea is obtained based on the bioactivity fingerprint comparison.

In an alternative embodiment, the invention provides a method for making pharmaceutical grade echinacea which comprises determining a total bioactivity of a representative aliquot using at least two bioassays selected from the group consisting of tyrosine kinase assay (TK). TNF-α. EGF tyrosine kinase. $p59^{fyn}$ tyrosine kinase and leukotriene D4.

The method of the invention is useful to make a pharmaceutical grade echinacea from an appropriate botanical material which has a given or desired biological activity. Preferably. the botanical material is an extract made from echinacea plant material such as an aqueous or organic extract such as an alcoholic extract or a supercritical carbon dioxide extract or organic solvent extract which may be subject to further processing. Alternatively, the botanical material is a powdered echinacea plant material. a seed oil. an essential oil or the product of steam distillation. In one embodiment. the botanical material is a homogeneous material in a single physical state. *e.g.* an oil or a solution. The botanical material may be a pure material derived solely from the botanical of interest.

In this invention the active component(s) include. but are not limited to. one or more of the following chemical classes: acetogenins. alkaloids. carbohydrates. carotenoids. cinnamic acid derivatives. fatty acids. fatty acid esters, flavonoids. glycosides. isoprenoids, lipids, macrocyclic antibiotics. nucleic acids. penicillins. peptides. phenolics. polyacetylenes. polyketides. polyphenols. polysaccharides. proteins. prostaglandins. steroids and terpenoids. Preferably, the active component is selected from the group consisting of cinnamic acid derivatives. polyketides. polyphenols. polysaccharides, proteins, prostaglandins. steroids and terpenoids. More preferably, the active component is selected from the group consisting of chlorogenic acids. echinosides. alkylamides, chicoric acids, caffeic acids and dienoic amides.

In an alternative embodiment, echinacea may be combined with one or more botanical materials selected from: aloe, Asian ginseng. astragalus. bilberry. black cohosh, burdock. chamomile. chestnut. coriolus versicolor. couchgrass, crampbark. dandelion root, dong quai. echinacea. elecampane, evening primrose. eyebright, false unicorn root,

feverfew, garlic, ginger, ginkgo, goldenseal, gota kola, grape seed extract, green tea, guggulipid, hawthorn, hops, ivy, kava, licorice, milk thistle, mistletoes (American, Asian and European varieties), motherwort, oats, osha, passion flower, pumpkin, pygeum, red clover, rosemary, Siberian ginseng, sarsaparilla, saw palmetto, skullcap, St. John's wort, stinging nettle, valerian, wild indigo, wild yam, and yerba mansa. The methods of the present invention for making pharmaceutical drugs encompass methods for PharmaPrinting™ echinacea plus one or more of the botanicals listed above as well as pharmaceutical grade drugs containing echinacea and one or more of the botanicals listed above. In one embodiment, the echinacea may be combined with elecampane, goldenseal, osha, wild indigo, yerba mansa, St. John's Wort or valerian.

By way of illustrative example, but not by way of limitation, pharmaceutical grade echinacea may be combined with a pharmaceutical grade botanical material such as valerian, goldenseal or St. John's wort. See U.S. patent application, serial No.08/956,615, entitled "PHARMACEUTICAL GRADE VALERIAN", filed October 23, 1997, incorporated in its entirety by reference herein; see also U.S. patent application, serial No.08/956,602 (attorney docket 9117-0014), entitled "PHARMACEUTICAL GRADE ST. JOHN'S WORT", filed October 23, 1997, incorporated in its entirety by reference herein. See also U.S. patent application, serial No. 08/838,198 entitled "PHARMACEUTICAL GRADE BOTANICAL DRUGS", filed April 15, 1997 with respect to goldenseal, on pages 164-166, Example 25, incorporated in its entirety by reference herein.

The bioactivity/clinical indication for the echinacea may be associated with a disease, disorder or condition of humans or other animals. Thus the methods are useful to produce pharmaceutical grade echinacea for treatment and/or amelioration and/or prevention of human and/or veterinary diseases, disorders or conditions. Exemplary indications include, but are not limited to, an allergic/ inflammatory disorder, a cancer, an endrocrine disorder, or a disorder induced by a microbial organism or a virus.

In these methods, the aliquot may be separated into both biologically active and inactive components. Furthermore, the marker fractions may comprise a class of related components.

This invention also provides a method of preparing a PharmaPrint® for a pharmaceutical grade echinacea. Furthermore, this invention provides for a pharmaceutical grade echinacea prepared by the methods described above.

3.1. DEFINITIONS

The term "pharmaceutical grade" when used in this specification means that certain specified biologically active and/or inactive components in a botanical drug must be within certain specified absolute and/or relative concentration range and/or that the components must exhibit certain activity levels as measured by a disease-, disorder- or condition-specific bioactivity assay. The disease, disorder or condition may afflict a human or an animal.

As will be understood by those skilled in the art, the term "pharmaceutical grade" is not meant to imply that the botanical drug is applicable only to products which are regulated for example those provided under prescription, *i.e.*, "Rx" products or over the counter, *i.e.*, "OTC". The term is equally applicable to products provided Rx. OTC or as a dietary supplement. *i.e.*, "DSHEA".

As used herein "components" means discrete compounds (*i.e.* chemicals) which either are present naturally in a botanical drug or have been added to the botanical drug so as to prepare a pharmaceutical grade botanical drug having components within a defined bioactivity range(s) and/or compositional range(s).

As used herein "active components(s)" means one or more component(s) for which the summation of the individual component(s) activity in a disease-specific bioassay accounts for a substantial portion of the observed biological activity of the botanical material. Preferably, the summation of the active components' activities accounts for the majority or greater than 50% of the observed biological activity.

As used herein "fractions" typically means a group of components or class of structurally similar components having defined parameters such as solubility, molecular weight range, polarity range, adsorption coefficients, binding characteristics, chemical reactivity or selective solubility. Most frequently fractions will be the product of selective solvent solubility and partition techniques (i.e. liquid-liquid extraction) including pH dependent separations, chromatographic separation techniques, *i.e.*, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, partition chromatography, preparative thin layer chromatography, affinity chromatography, size exclusion chromatography, liquid-liquid chromatography *e.g.*, counter-current chromatography or centripetal or centrifugal chromatography.

The present invention may be understood more fully by reference to the detailed description of the invention and examples of specific embodiments in Sections below and the appended figures.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of a procedure in accordance with the present invention which is used to establish standard chemical and/or bioactivity fingerprints against which subsequent processed botanical materials are compared during production of pharmaceutical grade drugs.

FIG. 2 is a schematic representation of a procedure in accordance with the present invention which is used to process botanical materials into pharmaceutical grade drugs.

FIG. 3 is a schematic representation of a procedure for isolating different classes of biologically active components.

FIG. 4 shows the structure of a variety of alkylamides found in echinacea.

FIG. 5 shows the structure of a variety of echinacides found in echinacea.

FIG. 6 shows the structure of a variety of caffeic acids found in echinacea.

FIG. 7 shows the chemical composition of several commercially available echinacea products sold in capsule form. Brands A, D, E and F are in the form of plant powders and brands B and C are in the form of plant extracts.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1. METHODS OF PHARMAPRINTING™

The present invention provides a method for producing botanical drugs which may be classified as being of pharmaceutical grade. The method is designated PharmaPrinting™. The pharmaceutical grade botanical drugs made by the method of the present invention are particularly well-suited for use in clinical studies and more importantly for use in treatment of patients. The method insures that the drug being used for a particular protocol will be of consistent quality and consistently suitable for use as human and veterinary prophylactic or therapeutic agents.

The present invention provides the ability to closely control the quality, dosage and clinical effectiveness of botanical extracts and other botanical materials, *e.g.*, botanical extract and mammalian tissue derived biological preparation. One aspect of the present invention involves the establishment of the chemical and/or bioactivity fingerprint standards

for various botanical materials. Once established, the fingerprint standards are used in drug production procedures to insure that the botanical materials meet pharmaceutical grade requirements. Specific quantitative and biological fingerprints are presented which have been established for a number of botanical materials as a further aspect of the invention. These fingerprints are useful for determining if a particular botanical material meets levels of pharmacological activity and composition requirements for a particular treatment regimen. Such a determination is important to insure that clinical studies and patient treatment with the botanical materials are based on consistent and verifiable extract composition parameters. This invention is useful in providing botanical materials which are sufficiently characterized and whose compositions are consistent between batches, so that they can be precisely dosed and used effectively in clinical settings. The methods described herein provide an assurance that the results of a clinical trial will be reproducible.

Initially, a sample of the botanical material of interest is obtained. Many botanicals are commercially available as the raw material or as a processed extract. Often it is a botanical extract or other composition which is intended for use as a drug. The processed material may include a plurality of active components which exhibit a given biological activity and plurality of inactive components which do not directly exhibit the biological activity of interest. In one embodiment, an aliquot is removed from the botanical material and subjected to a quality assurance or standardization procedure. Preferably, the aliquot is a representative aliquot of a homogeneous botanical material. The procedure involves separating the aliquot of botanical material into a plurality of marker fractions wherein each of the marker fractions includes at least one of the active components or in some cases one of the inactive components. The amount of active component or inactive component in each of the marker fractions is determined in order to provide a quantitative fingerprint of the aliquot. The degree of biological activity for each of the marker fractions is also determined to provide a biological activity fingerprint for the aliquot. The chemical and/or biological activity fingerprints of the aliquot are then compared to corresponding fingerprints which have been established for a pharmaceutical grade drug. If the fingerprints of the botanical match the standard fingerprints, then the botanical is identified as a pharmaceutical grade botanical drug. If not, then the botanical may be modified so as to provide a match with the standard fingerprints or may be rejected.

5.1.1. METHODS OF DEVELOPING A PHARMAPRINT®

The method of developing a PharmaPrint® for a botanical when a range of putative active components is known begins with a literature review. It involves reviewing the

chemical literature, the biological literature, the published bioassays and clinical data for the botanical. Particularly useful sources of information are the NAPRALERT computer database managed by Dr. Norman Farnsworth in the Program for Collaborative Research in the Pharmaceutical Sciences, University of Illinois, Chicago; Leung and Foster, Encyclopedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics, 2nd Ed. John Wiley & Sons: New York, NY, 1996; Herbal Drugs and Phytopharmaceuticals, ed. N.G. Bisset, CRC Press: Boca Raton, FL, 1994; Duke, Handbook of Biologically Active Phytochemicals and Their Activities, CRC Press: Boca Raton, FL, 1992; Tyler and Foster "Herbs and Phytomedicinal Products" in Handbook of Nonprescription Drugs Berardi et al. eds., United Book Press, Inc.: Washington, DC, 1996. For a given indication, the literature must be studied to confirm that the putative active components are actually associated with that disease state. In addition, if there are any bioassays known for the putative active components and known for the indication, the bioassays must be consistent with both the indication and the putative active components. The appropriate bioassay(s) is tied to a clinically relevant endpoint(s). The bioassay(s) should be quantitative over a wide concentration range. Typically, an IC_{50} curve (Inhibitory Concentration 50%), EC_{50} (Effective Concentration 50%), or an appropriate K_i or K_d (dissociation constant of the enzyme and its inhibitor) curve is prepared. A thorough chemical and biological analysis of both putative active components and chromatographic fractions of the botanical is then performed. The results are analyzed to prepare a quantitative analysis of the biological activity for each of the chemical components in the sample. Then, the bioactivity of the sample as a whole is compared to the bioactivity of the individual components. At this point the individual chemical components can be correlated with a clinically relevant endpoint. Similar methodologies may be applied to bioassays measuring stimulatory or inhibitory effects.

Based on activity of the components individually and knowing the total activity, the components should, when combined, account for a substantial portion of the biological activity. Generally, the combined activity will account for at least 25% of the total activity.

Preferably, the summation of the individual active components' activities account for the majority or greater than 50% of the observed biological activity. More preferably, the isolated individual components are responsible for more than 70% of the activity. More preferable still, the isolated individual components are responsible for greater than 80% of the biological activity.

Another consideration will be to select as few active components as possible to be part of the PharmaPrint™. Fewer active components are important for practical

considerations in raw material acceptance and manufacturing. In this invention, a correlation is established between the relevant chemical components and the bioactivity. Once a satisfactory correlation is established, it may not be necessary to perform the biological fingerprints on each sample. Rather, a chemical analysis of the appropriate components and/or marker fractions of each sample of the botanical of interest will suffice to account for most of the biological activity and establish that a given botanical sample is pharmaceutical grade.

In one embodiment, the present invention may involve one of the following procedures. One procedure, as schematically outlined in FIG. 1, involves establishing the compositional and bioactivity fingerprint standards for a given pharmaceutical grade botanical drug. Once the fingerprint standards are established. then the actual processing of the botanical into a pharmaceutical grade drug can be carried out as schematically outlined in FIG. 2.

The initial step in establishing the chemical and/or bioactivity fingerprint for a given botanical involves separating the extract or powder into one or more groups as represented by step 1 in FIG. 1. These groups are separated out and identified based on their potential as markers (which may or may not comprise active components) for the fingerprint which is to be established for the processed botanical material. The putative components or groups of putative components which are chosen and identified as potential markers will vary widely depending upon the botanical being processed and the pharmaceutical use. There should be at least two putative markers selected for each botanical. The number of potential markers may be more than five and can be as high 15 to 20 or more for complex botanical extracts or powders. The potential markers are identified and selected, for the most part. based on their potential biological activity or contribution to biological activity for a given pharmaceutical application. For a different indication the same botanical may be used for preparing an extract with a different extraction procedure in order to optimize specific bioactive constituents. Markers which have no apparent biological activity by themselves may be separated out and may be included as markers for use in the fingerprint. These "proxy" markers may be desirable as an internal standard where the markers' presence is indicative of other active components necessary to provide a substantial portion of the overall observed biological activity for the botanical drug. They also help to assure proper botanical identity of the drug (i.e. chemotoxonomy).

The initial separation of the botanical into various groups of putative markers is accomplished by conventional separation techniques ranging from simple extraction and partition, to complex affinity chromatographic techniques, including gel filtration

chromatography, flash silica gel chromatography and reverse phase chromatography. Once the putative markers have been identified for a given botanical, then the bioactivity of each of the markers is determined as depicted by step 2 in FIG. 1. The particular bioassay used to determine bioactivity of the botanical is chosen based upon the intended use for the botanical. The bioassay preferably will provide a reflection of the putative markers' bioactivity with respect to the condition or indication which is to be treated with the botanical.

The bioassay results obtained in step 2 are used to identify the components having the desired bioactivity (step 3) and those which are less active or essentially inactive (step 4). Each of the groups identified in steps 3 and 4 is then analyzed quantitatively to determine the amount of each identified component present in each group. The results of the bioassays and quantitative compositional assays are then used to prepare a bioassay fingerprint and/or a chemical fingerprint for the botanical as depicted by step 5 in FIG. 1. As part of establishing the fingerprints for the botanical. acceptable ranges of bioactivity and/or chemical composition are determined. This is done primarily based upon establishing acceptable ranges of bioactivity and quantitative amounts for each marker which provide for the desired pharmacological activity of the processed material as a whole.

In addition. various combinations of active and inactive marker fractions may be evaluated to establish potential increases in desired bioactivity resulting from combinations of the active and inactive components.

The bioassay and quantitative fingerprints which are established in step 5 provide an accurate identification of the botanical which can be used in establishing the dosage regimens and treatment schedules which are necessary for clinical use. The dosage regimens and treatment schedules are established using conventional clinical methods which are commonly employed when investigating any new drug. The processed material which is used to determine the dosage and treatment schedules must be matched with and meet the requirements of the fingerprints established in step 5. This method insures that the dosage and treatment schedules are effective and reproducible since the processed materials used in the dosage and scheduling studies all have the same fingerprints in accordance with the present invention.

The bioassay and quantitative fingerprints which are determined by the general procedure as set forth in FIG. 1 are used as part of the manufacturing procedure for producing pharmaceutical grade botanical drugs. The fingerprints are used as part of a quality assurance or standardization procedure to insure that a given botanical contains the

appropriate compounds and is processed correctly to provide a botanical drug which will perform the same clinically as the material which has been standardized and tested in accordance with the procedure set forth in FIG. 1.

An exemplary procedure for producing pharmaceutical grade botanicals in accordance with the present invention is shown schematically in FIG. 2. The botanical of interest 21 is first processed by extraction, powdering or other manufacturing process to form a processed botanical material 22. A sample of the processed material 22 is then analyzed to establish whether or not it matches the fingerprint requirements established during the standardization procedure of FIG. 1. This quality assurance or standardization procedure is depicted at 23 in FIG. 2. If the processed material meets the previously established fingerprint requirements for the particular material, then it is approved as being of pharmaceutical grade as represented by step 24. If the material is close, but does not quite match the standard fingerprint, then it is modified as required to match the fingerprint standards (step 25). The modification of the processed material to meet fingerprint standards may be done by a variety of ways. The methods of further processing botanicals may including additional extraction of the botanical, selective extraction, selective processing, recombination of batches (*e.g.* mixing high and low dose batches to prepare the pharmaceutical grade material) or the addition of various compounds, as required. If the botanical is substantially outside the fingerprint ranges for both bioactivity markers and quantitative markers, then the batch is rejected (step 26).

In one embodiment, the quality assurance standardization step 23 used to determine if a given botanical is pharmaceutical grade involves obtaining a uniform sample, preferably a homogeneous sample, or aliquot of the botanical which is to be tested. The sample should include the active components which contribute to the observed biological activity of the material and produce the bioactivity and/or chemical fingerprint of the previously determined standard. The sample will also include one or more inactive components. Inactive components are those which may not have a direct measurable biological activity. Inactive components include the following categories: components with activity so low that they do not account for a substantial portion of the activity; components whose presence indicates the presence of other bioactive components and can act as proxy markers for these components; inactive components that are chemically or biologically inactive in the relevant assays. The sample is preferably only a small aliquot of the botanical material being tested. Accordingly, it is important that a uniform sample, preferably a homogeneous sample, be obtained which is representative of the entire batch of material.

A more detailed schematic is shown in FIG. 3 showing the initial separation of the different components present in an aqueous extract of a botanical. Sequential extraction and precipitation are used to isolate the active components in either the aqueous or the organic phase. The scheme in FIG. 3 is particularly well suited for separating the classes of water-soluble active components from a botanical such as mistletoe.

An exemplary general method for separating plants into major classes of chemical components is set forth schematically in FIG. 3. Primarily fresh plants (including leaves, roots, flowers, berries and stems) should be used, although dried materials may also be utilized. Specific plant parts, such as the leaves, flowers, stems or root may be used if desired.

In this method the specific part or whole plant may be frozen at liquid nitrogen temperature. This facilitates grinding and also preserves the integrity and potency of the active components.

The pulverized powder is extracted with distilled water repeatedly. If desired, the extraction may be carried out with hot water, alcohol, other organic solvents, aqueous alcohol, dilute acetic acid or any combination thereof. The actual temperature chosen is preferably close to or at the boiling temperature of water. It is preferred that the overall bioactivity of the extract be initially determined. The combined extracts are subjected to a specific bioassay, *e.g.*, a test for inhibiting the growth of bacteria in Petri dishes if the drug is to be used as an antibacterial. Alternatively, tests against cell cultures of cancer cells are conducted preferably if the drug is intended for use as an anticancer agent. From these data, bioactivity units contained in an extract per ml are calculated (bioactivity units are defined as the dilution number of this extract needed to inhibit 50% growth of bacterium or cancer cell in test system). Similarly bioactivity units for a stimulatory effect, *e.g.*, immunostimulation can be calculated.

For establishing a pharmaceutical fingerprint (PharmaPrint®) in accordance with the present invention, the plant is extracted according to the procedure as set forth in FIG. 3 to separate it into major components (*e.g.* saponins, terpenoids, lipids, alkaloids, nucleic acids, proteins and carbohydrates). Each separated group of components is tested for bioactivity as needed. This may point to activity (*e.g.* in protein and alkaloid fractions as in *Viscum album*). The active class or classes of compounds are further separated into individual components by affinity chromatography, high performance liquid chromatography, gas chromatography or other chromatography. The components with major contribution towards biological activity are quantified on the basis of weight and specific bioactivity units. These components provide the fingerprint to establish the pharmaceutical

requirements for the original herbal extract. The bioactivity units per ml of the pharmaceutical grade extract provide a way to establish exact dosage for clinical studies.

Once the sample is separated into individual marker fractions, and at least one having at least one active component, each fraction is analyzed to determine the amount of active component therein and provide a quantitative fingerprint of the sample. The quantitation of each fraction can be achieved using any of the known quantitative analysis methods. Exemplary quantitation methods include gravimetric analysis, spectral analysis or the use of quantitative detectors, such as those used in gas chromatography or high performance liquid chromatography and other separation systems. Other suitable quantitative analytical methods include analysis by enzymatic, radiometric, colorimetric, elemental analysis spectrophotometric, fluorescent or phosphorescent methods and antibody assays such as enzyme linked immunosorbant assay (ELISA) or radioimmunoassay (RIA).

In one embodiment, the results of the quantitative analysis of each fraction are used to prepare a quantitative fingerprint of the sample. The fingerprint is composed of the quantity of component in each of the marker fractions and the identity of the component. This quantitative fingerprint is then compared to the known standard fingerprint which has been established (FIG. 1) in order for the material to be considered as pharmaceutical grade. If the quantitative fingerprint of the sample falls within the range of quantities set forth for the pharmaceutical grade fingerprint, then the material may be identified as being of pharmaceutical grade.

As a further part of the quality assurance assay, the individual marker fractions may be subjected to biological assays. The biological assays which are used to test the various fractions are the same as those used for the standard fingerprint and will also depend upon the particular clinical use intended for the material.

The bioactivity fingerprint generated for the material is compared to the standard bioactivity fingerprint which has been established in order for the material to be considered as pharmaceutical grade. If the bioactivity fingerprint of the sample falls within the range of bioactivities set forth for the pharmaceutical grade fingerprint, then the material is identified as, and approved as, being of pharmaceutical grade.

5.1.2. ALTERNATIVE METHODS OF DEVELOPING A PHARMAPRINT®

The method of developing a PharmaPrint® for a botanical when the putative active components are not known also begins with a literature review. It involves reviewing any chemical literature, biological literature, published bioassays or clinical data available for the botanical, or related botanicals, or for botanicals with related activities. Based on the

disease state, a series of relevant bioassays is chosen. The activity of the total sample or extract is analyzed using bioassays. Those bioassays that show activity are then used to analyze fractions of the botanical for which the putative active components are not yet known. The fractionation is based on the usual methods, *e.g.*, separation by dielectric constant, biological affinity, polarity, size, solubility or absorptive power. The fractions are then analyzed to determine which fraction is responsible for the activity. Assuming activity is found, each active fraction is refractionated to isolate the individual putative active components, *i.e.*, pure chemical compounds. Based on knowing the individual chemical compounds and knowing their quantitative biological activity, a quantitative potency curve may be drawn and the 50% inhibitory concentration (IC_{50}) for each individual chemical component may be determined. If the putative active components are agonists, then other parameters (binding, activation, response) may be needed. In the general case, the bioassay will consist of appropriate tests of the stimulatory or inhibitory effects of the constituents, fractions or entire extract, followed by an appropriate quantitative evaluation of those effects. For the most likely (or typical) assays in which a standard (or radiolabelled) agonist or antagonist causes a measurable effect, inhibition and/or stimulation by the subject material may be assessed and expressed typically via the determination of an IC_{50}, EC_{50}, etc. value, or other suitable measure (*e.g.*, K_i, K_d, K_m, etc). The activities of individual putative active components are then totalled and that summation is compared to the activity in the unfractionated botanical sample. If these components account for a substantial portion of the activity, then one has an initial fingerprint of "active components" for the botanical where the active components were not known.

5.1.3. ADDITIONAL VARIATIONS ON THE METHOD OF DEVELOPING A PHARMAPRINT®

The general method outlined above for PharmaPrinting™ a botanical whose putative active components are not known has several variations should complications arise in the course of the analysis. One variation occurs when the summation of individual components do not account for a substantial portion of the biological activity of the botanical. At this point there are several likely reasons for the reduced activity of the individual components, one, decomposition or degradation of active components or, two, a synergistic effect. In another possible scenario there may be no significant or greatly lessened activity seen from any of the fractions, but the whole botanical or extract shows activity in the bioassay. Nonspecific matrix effects may also lessen the total extract activity, when compared to standards.

To determine if the active components are decomposing in the course of the assay is relatively simple. One merely recombines all of the fractions and compares the activity of the recombined fractions with the activity of the crude material. If substantial activity has been lost, then the problem is probably decomposition. To determine which active components may be decomposing, the chromatographic analysis of the crude botanical is compared with that of the recombined fractions. Peaks that are missing or are reduced in size indicate that components may be decomposing. To overcome decomposition many methods exist. Typically, milder extraction/fractionation methods such as liquid-liquid chromatography (counter-current chromatography) or supercritical carbon dioxide extraction or chromatography may be used.

Another explanation for the activity of the individual fractions not accounting for a substantial portion of the expected total activity is a synergistic effect between one or more active components with each other, or inactive components. To determine that a synergistic effect is taking place, pair-wise recombined fractions need to be analyzed. If the combined fractions show more activity than the individual fractions, two or more individual components in the fractions may be acting synergistically. For example, one may have three fractions, each alone responsible for 10% of the bioactivity (i.e., their uncombined additive bioactivity is 30%) but combined responsible for 100% of the activity. In that case the fractions are acting synergistically. By repeated pair-wise recombination of fractions or looking at larger fractions, any synergistic activity will be discovered. Once two fractions show synergy, they are then refractionated as above, and pairs of individual fractions or pairs of isolated components are studied to find the individual components that act synergistically. Three way comparisons of individual components or fractions may also be studied.

What if the fractions have no activity in the bioassay in which the botanical shows activity? Here, the explanations include decomposition, synergy, or many active components such that no individual fraction shows activity. The first step would be to fractionate each initial fraction and see if active components appear in the bioassay. If that does not succeed, the fractions should be recombined and assayed to determine if decomposition of the actives is taking place. If decomposition is taking place, the appropriate measures as described above should be taken. If there is no decomposition, then alternative methods of fractionation should be tried. Eventually, large enough or appropriately sized or selected fractions will show activity. If synergy is a suspected problem, then proceed as in the synergy section described above.

5.2. METHODS OF PROCESSING AND EXTRACTING BOTANICAL MATERIALS

The botanical material may be processed to form an aqueous or organic extract of the whole plant or a selected part of the plant. The botanical material can also be processed in whole or part to form a powder. Many of the botanicals of interest are commercially available as powders, aqueous extracts, organic extracts or oils. In one embodiment, extracts of the plant material are preferred because they are easier to dissolve in liquid pharmaceutical carriers. However, powdered plant materials are well-suited for many applications where the drug is administered in solid form, *e.g.*, tablets or capsules. Such methods are well known to those of skill in the art. Furthermore, many of the plant materials and/or extracts are available commercially. As examples of the processing and extracting of botanicals the following examples are provided. Additional examples are provided in the detailed description.

For a typical root, it may be sliced, frozen or pulverized. If powdered it is then shaken with an appropriate solvent and filtered (Tanabe et al., 1991, *Shoyakugaku Zassi*, 45(4):316-320). Alternatively, the following methods are used: the root is homogenized, acetone extracted and filtered; the botanical may be steam distilled to obtain essential oils and the distillate dissolved in acetone-water or appropriate solvent; or the cut rhizomes are frozen and/or freeze-dried and the resulting powder acetone-water extracted (Tanabe et al., 1991, *Shoyakugaku Zassi* 45(4):321-326). Another method of processing botanicals is aqueous extraction with 100°C water (Yamahara et al., 1985, *J. Ethnopharmacology* 13:217-225). The initial solvent extract from the methods above may be further extracted using liquid/liquid extraction with an appropriate solvent. The botanical may be extracted in two steps using polar and non-polar solvents respectively. The solvents are then evaporated and the fractions combined (Nagabhusan et al., 1987, *Cancer Let.* 36:221-233). Botanicals may also be processed as a paste or powder which may be cooked (Zhang et al., 1994, *J. of Food Science* 59(6):1338-1343).

A variety of solvents may be used to extract the dried botanicals, for example acetone, acetonitrile, dichloromethane, ethyl acetate, ethanol, hexane, isopropanol, methanol, other alcohols, and supercritical carbon dioxide (Sipro et al., 1990, *Int. J. of Food Science and Technology* 25:566-575 and references therein).

For other botanicals such as Saw Palmetto, the medicinal products are the seed oil or dried berries. In a typical preparation, a hexane or supercritical carbon dioxide extract is prepared. Many Saw Palmetto preparations are commercially available, for example Permixon™ or Talso™. For an example of supercritical carbon dioxide extraction of a

botanical, see Indena, European Patent No. 0 250 953 B1. Alternatively, the botanical may be crushed and extracted with an appropriate solvent (90%) in a soxhlet (Elghamry et al., 1969, *Experientia* 25(8):828-829). The botanical may also be ethanol extracted (Weisser et al., 1996, *The Prostate* 28:300-306).

The dried material may be prepared in a variety of ways including freeze-drying, drying via microwave, cooling with liquid nitrogen and pulverizing; drying at 70°C under vacuum for a duration of 10 hours; or air-drying in the shade, or with forced heated air (List and Schmidt, Hagers Handbuch der Pharmazeutischen Praxis, Springer-Verlag: New York, 1993, 1973-79; Araya et al., 1981, *Journal of Comparative Pathology*, 135-141). Teas, dilute aqueous extracts, also known as infusions, may be made in 60-100°C water (Nosel and Schilcher, 1990). Decoctions may also be utilized. Extraction is more efficient when the particle size is less than .25 mm (List and Schmidt, Phytopharmaceutical Technology, CRC Press: Boca Raton, FL, 1989).

Various guidelines are available for preparing oil extracts of botanicals. The botanical may be digested (macerated) in oil at 45°C for 10 days, while others recommend 70°C for 12-24 hours (Hobbs, 1989, *HerbalGram* 18/19:24-33; Smith et al., Quality Validation Laboratory - Herb Pharm: Williams, OR, 1996). In St. John's Wort for example, exposing the preparation to sunlight during the extraction process has been reported to result in a four-fold increase in flavonoid content calculated as quercetin (Maisenbacher and Kovar, 1992). Additionally, for St. John's Wort, two-fold increases of hypericin have been reported in oil preparations in which the material has been further extracted with alcohol, and mixed with the oil (Georgiev et al., 1983, *Nauchni Tr.-Vissh Inst. Plovid.* 30:175-183).

Alternatively an alcohol-water preparation may be prepared of the botanical (Dyukova, 1985, *Farmitsiya* 34:71-72; Georgiev et al., 1985, *Nauchni Tr.-Vissh Inst. Plovid.* 32:257-263; Wagner and Bladt, 1994, Kowalewski et al., 1981, *Herba Pol.* 27:295-302). According to Hagers Handbuch a tincture of a botanical, such as St. John's Wort, may be prepared by using drug or freezing ethanol soaked botanical materials, and filtering and preserving in dark bottles (List and Hörhammer, 1993).

Some botanicals, such as St. John's Wort, are both temperature and light sensitive. For this type of botanical the material should be dry packed with a refrigerant or shipped under refrigeration and protected from light and air. In St. John's Wort, hypericin content has been shown to drop significantly in powdered extract, tablet and juice preparations when stored at temperatures of 60°C-140°C for more than six weeks. Dry extracts stored at 20°C were found to remain stable for at least one year (Adamski et al., 1971, *Farm. Pol.* 27:237-241; Benigni et al. Hypericum. Plante Medicinali: Chimica, Farmacologia e

Terapia, Milano: Inverni & Della Beffa; 1971). Other St. John's Wort constituents, hyperforin and adhyperforin found in oil preparations are highly unstable, especially when exposed to light, and can degrade in as little as 14 days (Meisenbacher et al.. 1992, *Planta Med.*, 351-354). Stability (in absence of air) was increased to six months in a preparation extracted with ethanol. Similarly, up to four xanthones and several flavonoids including quercetin and I3', II8-biapigenin have been detected suggesting these may be among the active constituents in external preparations (Bystrov et al., 1975, *Tetrahedron Letters* 32:2791-2794).

For fingerprinting echinacea in accordance with the present invention, green leaves and roots are cut and frozen at less than -100°C. The mixture is then pulverized and extracted with a known volume of water. This procedure is preferred to retain the maximum amounts of components. The components are a mix of volatile oils, glycosides, amides and polyacetylenes.

5.2.1. POWDERED PLANT MATERIALS AND LIQUID EXTRACTIONS

One common form of liquid extract of botanical material are teas. Teas may be prepared through processes of infusion or decoction. They are generally an effective means to extract water soluble components from dried or fresh botanicals.

Another common form of liquid botanical extract is a tincture. A botanical tincture is typically an alcoholic or hydroalcoholic solution prepared from a fresh or dried botanical. It is usually prepared through a process of percolation or maceration.

Tinctures of potent botanicals, and homeopathic mother tinctures, may represent 10 g of botanical (dry weight) in 100 ml of tincture. Most other botanicals have 20 g of botanical represented in 100 ml of tincture. The respective ratios of dried botanical to solvent for these preparations are 1:10 and 1:5, respectively. While these concentrations have been officially recognized by the U.S. National Formulary it has become common for tinctures to be prepared in 1:4, and other concentrations.

As compared to crude botanical extracts, tinctures may have a reduced microbial load and longer shelf life. This is largely due to the presence of alcohol at 20% or greater concentrations in the extract. Occasionally liquid extracts are made with glycerin and water as the solvent. These glycerites usually need to have at least 50% glycerin present to inhibit microbial contamination. Glycerites may also be prepared from tinctures by evaporating off alcohol and "back adding" glycerin in its place.

Another type of liquid extract are fluid extracts. Fluid extracts are liquid preparations of botanicals that represent the medicinal properties of 1 g of dried botanical in 1 ml of extract. Official versions are made by the percolation process according to official monographs which determine the solvent to be used.

Liquid extracts that are concentrated, usually through evaporation of the solvent, may form extracts that are semi-solid in nature. Dry powdered extracts may be prepared by the absorption of liquid extracts onto suitable carriers before solvent removal.

5.3. SEPARATION OF FRACTIONS

Once the sample extract has been prepared and/or alternatively purchased as a commercially available extract, a portion needs to be subjected to fractional analysis. If the fingerprint has already been established, the sample or aliquot is separated into the same plurality of marker fractions which are present in the standard fingerprint. Each of the marker fractions will include one or more of the active or inactive components. The marker fractions are established on an individual basis for each botanical material being tested. For some materials only a few marker fractions are required. For other more complex materials, there may be numerous marker fractions. For example in mistletoe, *Viscum album* L. protein extract, the preferred protein marker fractions are those fractions which are separated based on the sugar binding affinity of the fraction. However, different parameters for identifying and separating the materials into the marker fractions may be established based upon the types of components present in the botanical material. Separation of the sample into the marker fractions may be accomplished by any of the conventional separation techniques including liquid chromatography and extraction procedures. The same procedures which were used to establish the standard fingerprints should be used. Since the various fractions may be tested for biological activity, it is preferred that non-destructive separation techniques be utilized. Liquid column chromatography is a useful separation technique with affinity chromatography based on the specific binding ability of the compounds (*e.g.*, carbohydrates and target enzymes) being particularly used.

After the fractionation, the solvent is removed and the material is dissolved in an appropriate medium for the bioassays. Examples of appropriate media include DMSO, ethanol, various detergents, water and an appropriate buffer. The choice of solvent will depend on the chemical nature of the component being analyzed and the compatibility with the assay system.

5.4. ESTABLISHMENT OF APPROPRIATE BIOASSAYS

Exemplary biological assays may include any cell proliferation assays, such as the measurement of L 1210 cell inhibition, immune activity or inhibition of critical enzyme which relates to specific diseases. Examples of other transformed cell lines which can be used for bioassays include HDLM-3 Hodgkin's lymphoma and Raji Burkitt's lymphoma, hepatoma cell line, primary or established cultures of human/animal cell lines which carry specific cell receptors or enzymes.

The results of the biological assays are used to prepare a bioactivity fingerprinting of the material. The fingerprint can be as simple as an assay of two selected marker fractions. Conversely, the fingerprint can include numerous different bioassays conducted on numerous different fractions. The same assay may be conducted on different marker fractions. Also, different assays may be conducted on the same marker fraction. The combination of bioassays will depend upon the complexity of the given botanical material and its intended clinical use. The bioassays will be the same as those conducted in establishing bioactivity fingerprint of the standard material.

5.4.1. ENZYMATIC AND RECEPTOR BASED ASSAYS

Enzymatic and receptor based assays are preferable in the practice of this invention. Assays are chosen either based on accepted enzymatic assays for a clinical disorder or they are chosen from relevant assays for a given clinical disorder. It is important to choose appropriate bioassay that may be validated. Ideally, a bioassay should be rugged, that is reproducible over time and show a quantitative dose response over a wide concentration range. An issue faced with a botanical for which the active components are not known is the choice of a relevant bioassay. Here, the human therapeutic use will serve as a guide to pick assays known in the art based on possible mechanisms of action. The mechanism of action should be consistent with a clinically relevant endpoint. There are a wide array of clinically relevant assays based on enzymatic activity, receptor binding activity, cell culture activity, activity against tissues and whole animal *in vivo* activity.

This section will address enzymatic and receptor binding assays. There are many books on enzymatic or receptor assays, for example, Methods in Enzymology by Academic Press or Boyers, The Enzymes. Bioactive Natural Products, Detection, Isolation, and Structural Determination, S. M. Colegate and R. J. Molyneux, CRC Press (1993), also discusses specific bioassays. Methods in Cellular Immunology, R. Rafael Fernandez-Botran and V. Vetvicka, CRC Press (1995) describes assays from immune cell activation and cytokine receptor assays. "Screening Microbial Metabolites for New Drugs-Theoretical and

Practical Considerations" describes additional methods of finding pharmaceutically relevant bioassays (Yarbrough et al. (1993) *J. Antibiotics* 46(4):536-544). There are also many commercial contract research vendors, including Panlabs, Paracelsian and NovaScreen.

For example, for a botanical useful for treating neurological disorders, the array of bioassays might include adrenergic receptors, cholinergic receptors, dopamine receptors, GABA receptors, glutamate receptors, monoamine oxidase, nitric oxide synthetase, opiate receptors, or serotonin receptors. For cardiovascular disorders the array of assays may include adenosine A_1 agonism and antagonism; adrenergic α_1, α_2, β_1 agonism and antagonism; angiotensin I inhibition; platelet aggregation; calcium channel blockade; ileum contractile response; cardiac arrhythmia; cardiac inotropy; blood pressure; heart rate; chronotropy; contractility; hypoxia, hypobaric; hypoxia. KCN; portal vein, potassium depolarized; portal vein, spontaneously activated; or thromboxane A_2, platelet aggregation. For metabolic disorders the following bioassays may be used: cholesterol. serum HDL, serum total; serum HDL/cholesterol ratio: HDL/LDL ratios; glucose. serum - glucose loaded; or renal function. kaluresis, saluresis, and urine volume change. For allergy/inflammation disorders the following bioassays may be used: allergy, Arthurs reaction, passive cutaneous anaphylaxis; bradykinin B_2; contractility, tracheal; histamine H_1 antagonism; inflammation. carrageenan affects on macrophage migration; leukotriene D_4 antagonism; neurokinin NK_1 antagonism; or platelet activating factor, platelet aggregation or induction of biosynthesis of important inflammatory mediators (e.g. interleukins IL-1, IL-6, tumor necrosis factor or arachidonic acid). For gastrointestinal disorders the following bioassays may be used: cholecystokinin CCK_A antagonism; cholinergic antagonism, peripheral; gastric acidity, pentagastrin; gastric ulcers, ethanol: ileum electrical stimulation modulation; ileum electrical stimulation spasm or serotonin 5-HT_3 antagonism. For antimicrobial, antifungal, or antitrichomonal disorders the following are used: *Candida albicans*; *Escherichia coli*; *Klebsiella pneumonaie*; *Mycobacterium ranae*; *Proteus vulgaris*; *Pseudomonas aeruginosa*; *Staphylococcus aureus*, methicillin resistant; *Trichomonas foetus*; or *Trichophyton mentagrophytes*. For other indications, one of skill in the art would be able to choose a relevant list of bioassays.

Specific examples of assays based on enzymes or receptors include the following: acetyl cholinesterase; aldol-reductase; angiotensin converting enzyme (ACE); adrenergic α, β, rat androgen receptor; CNS receptors; cyclooxygenase 1 or 2 (Cox 1, Cox 2); DNA repair enzymes; dopamine receptors; endocrine bioassays, estrogen receptors; fibrinogenase; GABA A or GABA B; β-glucuronidase; lipoxygenases, *e.g.*, 5-lipoxygenase; monoamine oxidases (MAO-A, MAO-B); phospholipase A_2, platelet activating factor (PAF); potassium

channel assays; prostacyclin cyclin; prostaglandin synthetase; serotonin assays, *e.g.*, 5-HT activity or other serotonin receptor subtypes; serotonin re-uptake activity; steroid/thyroid superfamily receptors; thromboxane synthesis activity. Specific enzymatic assays are available from a variety of sources including Panlabs™ Inc (Bothell, WA) and NovaScreen™ (Baltimore, MD). Additional assays include: ATPase inhibition, benzopyrene hydroxylase inhibition, HMG-CoA reductase inhibition, phosphodiesterase inhibition, protease inhibition, protein biosynthesis inhibition, tyrosine hydroxylase and kinase inhibition, testosterone-5α-reductase and cytokine receptor assays.

5.4.2. CELL CULTURE AND OTHER ASSAYS

In addition to the enzymatic and receptor assays, there are also other biological assays. Preferably, these assays are performed in cell culture but may be performed on the whole organism. Cell culture assays include activity in cultured hepatocytes and hepatomas (for effect on cholesterol levels, LDL-cholesterol receptor levels and ratio of LDL/HDL cholesterol); anti-cancer activity against L 1210, HeLa or MCF-7 cells; modulating receptor levels in PC12 human neuroblastoma cells; modulation of primary cell culture activity of luteinizing hormone (LH), follicle stimulating hormone (FSH) or prolactin; Ca^{2+} influx to mast cells; cell culture assays for phagocytosis, lymphocyte activity or TNF release; platelet aggregation activity or activity against HDLM-3 Hodgkin's lymphoma and Raji Burkitt's lymphoma cells, antimitotic activity, antiviral activity in infected cells, antibacterial activity (bacterial cell culture) and antifungal activity. Tissue or whole animal assays may also be used including anti-inflammatory mouse ear dermatitis, rat paw swelling; muscle contractility assays; passive cutaneous anaphylaxis: vasodilation assays; or whole animal carbon clearance tests. These assays are available from a variety of sources including Panlabs™ Inc. (Bothell, WA).

5.4.3. ANTICANCER ACTIVITY

The anticancer effects of drug can be studied in a variety of cell culture systems; these include mouse leukemias, L 1210, P388, L1578Y etc. Tumor cell lines of human origin like KB, and HeLa have also been used. In a typical assay tumor cells are grown in an appropriate cell culture media like RPMI-1640 containing 10% fetal calf serum. The logarithmically growing cells are treated with different concentrations of test material for 14-72 hours depending upon cell cycle time of the cell line. At the end of the incubation the cell growth is estimated by counting the cell number in untreated and treated groups. The cell viability can be ascertained by trypan blue exclusion test or by reduction of tetrazolium

dyes by mitochondrial dehydrogenase. The ability of a drug to inhibit cell growth in culture may suggest its possible anticancer effects. These effects can be verified in animals bearing tumors, which are models for human disease (Khwaja, T.A., et al. (1986) *Oncology*, 43 (Suppl. 1): 42-50).

The most economical way to evaluate the anticancer effects of an agent is to study its effects on the growth of tumor cells in minimum essential medium (MEM) containing 10% fetal calf serum. The drug-exposed cells (in duplicates) are incubated in a humidified CO_2 incubator at 37 °C for 2-4 days, depending upon the population-doubling time of the tumor cells. At the end of the incubation period the cells are counted and the degree of cell growth inhibition is calculated from a comparison with untreated controlled cells grown under identical conditions. The type of cell lines used have varied from laboratory to laboratory depending upon individual needs. The National Cancer Institute (NCI) in the United States recommends the use of KB cells (a human nasopharyngeal carcinoma) for the evaluation of anticancer drugs *in vitro*. The cell growth inhibition is determined by estimating the protein content (Lowry's method) of the drug-treated and untreated controls. NCI has also recommended the use of suspension culture of mouse leukemia P388 for the evaluation of anticancer potential of plant extracts and related natural products.

Mouse leukemia L1210 cells, cultured in microtiter plates are routinely used for *in vitro* assays for anticancer activity. The cell population-doubling time of leukemia L1210 is 10-11 h and a drug exposure of 48 h (3-4 generations of logarithmic growth) is used for the evaluation of its antitumor activity. For growth inhibition studies all stock solutions and dilutions are made with sterile 0.9% NaCl solution. The cell cultures are seeded at 2-5 x 10^4 cells/ml in duplicates for each inhibitor concentration in a microtiter place (0.18 ml/well). The inhibitors are added in 0.02 ml volume to achieve 1:10 dilutions in each case. The covered microtiter plate is incubated for 48 h in a humidified CO_2 incubator containing 5% CO^2 in air. At the end of the incubation period aliquots of each well are added to a measured volume of isotonic saline and counted in an electronic cell counter. The cell viability is determined by trypan blue exclusion. The results are calculated by plotting percent cell growth inhibition (as compared to cell density of the saline-treated controls) versus log of drug concentration and expressed as the concentration which caused 50% inhibition in cell growth (IC_{50}) as determined from the graph.

The cytotoxic effects of a drug on a tumor cell line may also be evaluated. However, these experiments require longer periods of time to study and are more expensive. In these studies drug-treated cells are washed free of drug and then plated in soft agar or an appropriate medium and the cellular viability is estimated by the ability of the surviving

cells to multiply and form microscopic colonies. The number of cellular colonies obtained with certain drug concentrations is compared with those obtained from untreated controls to evaluate cell kill or cytotoxic activity. In studies with mistletoe extract we have used loosely adherent cultures of EMT-6 cells (a mouse mammary adenocarcinoma). These cells are grown in Eagle's MEM (F14) containing 10% dialyzed fetal calf serum and antibiotics. The cell suspension is spun and the pellet suspended in Spinner's medium supplemented with 10% dialyzed fetal calf serum (70 cells/ml), plated in plastic Petri dishes and incubated for 2 h to permit cells to attach. At this time cells are exposed to various concentrations of extract for 2-24 h. Then, the medium is removed and replaced with drug-free medium and the dishes incubated for 5-7 days. The colonies are stained with methylene blue (0.33% in 0.01% KOH) and counted with an automatic colony counter. The plating efficiency of EMT-6 cells is 46%. (Khwaja et al., 1986, *Oncology*, *43(Supp. 1)*:42-50).

5.4.4. ANTIVIRAL ACTIVITY

The antiviral activity of different drugs can be ascertained in cell culture of human cell lines like HeLa or H9 lymphoma cells. These cells are infected with virus and the virus is allowed to propagate in cell cultures. The ability of virus to produce cell lysis or cytopathic effects is taken as the end point. For example, HIV infection of H9 cells results in production of multinucleated cells. These cytopathic effects, if reduced or eliminated by certain concentrations of the drug, indicates its potential as an anti-HIV agent. These results can be validated by estimation of viral enzyme in the cell cultures. *e.g.*, by studying the amount of the expression of viral reverse transcriptase. A decreased expression of the viral enzyme would support antiviral effect of the drug treatment (Khwaja, T.A. U.S. Patent No. 5,565.200; J. Levy et al. 1984. *Science* 225: 840).

5.5. ANALYTICAL METHODS FOR ANALYZING CHEMICAL COMPONENTS

There are many methods to separate and analyze individual chemical components including gas chromatography (GC), mass spectroscopy (MS), GC-MS, high performance liquid chromatography (HPLC), HPLC-MS, thin layer chromatography (TLC), high performance TLC (HPTLC) gel chromatography and reverse phase chromatography (RPC). These chromatographic methods may be performed either on an analytical scale or a preparative scale. To determine the actual chemical structure of unknown components, nuclear magnetic resonance (NMR) and mass spectrum fragmentation analysis are typically used.

The determination of the type of chromatography will depend on the chemical components most likely responsible for the bioactivity. For example if the bioactivity is likely due to fatty acids, the fatty acids are esterified and the esters analyzed on a GC. For organic compounds with alcohol groups, they are modified to prepare ethers, silyl derivatives or other less polar functional groups. These derivatives are then suitable for analysis by GC (Steinke et al., 1993, *Planta Med.* 59:155-160; Breu et al., 1992, *Arzneim.-Forsch/Drug Res.* 42(1):547-551). If the activity is most likely due to flavonoids, HPLC is the method of choice. Reverse-phase HPLC (RP-HPLC) has been used to analyze flavonoids from a variety of botanicals, specifically hawthorn, passion flower, chamomile, ginkgo (Pietta et al., 1989, *Chromatographia* 27(9/10):509-512). Plant constituents have been quantitatively determined by TLC (Vanhaelen and Vanhaelen-Fastre, 1983, *J. Chromatography* 281:263-271) as well as MS-analysis for garlic. CRC Handbooks of Chromatography on "Analysis of Lipids". K. D. Mukherjee. "Analysis and Characterization of Steroids". H. Lamparczyk. J. Sherma. and "High-Performance Liquid Chromatography of Peptides and Proteins". C.T. Mant and R.S. Hodges. are available and describe columns and solvent systems.

5.6. ANALYSIS OF FRACTIONS

In an alternative embodiment. rather than base the pharmaceutical fingerprint (PharmaPrint®) on discrete chemical components of known bioactivity, one may also establish the PharmaPrint® using defined fractions or classes of compounds. Some chemical constituents in botanicals form such a complex mixture of closely-related components that. from a practical point of view. it is desirable to base the PharmaPrint® on fractions or classes of components rather than on individual components. Examples of these types of components are lectins (sugar-binding proteins) or glycoproteins as well as the silymarins in milk thistle. There are many examples of fractional analysis (Gel Filtration Principles and Methods Pharmacia Biotech, Rahms i Lund: Sweden; Utsumi et al., 1987, *J. Biochem.* 101:1199-1208).

5.7. METHODS OF USE OF PHARMAPRINTED™ MATERIALS

After the botanical material has an established fingerprint, individual samples are then analyzed to determine if they fall within the accepted standards. Once the sample has been approved it is suitable for a variety of diseases relevant to humans and animals. Such materials are useful in clinical trials so as to provide materials of consistent quality and precise dosage formulations for trials. The PharmaPrinted™ material is also useful for

toxicological tests in animals where once again the consistency of the material is useful for quantitative toxicological analysis. In many cases it would be of use as a reference material for analytical or biological use.

The PharmaPrinted™ botanical materials are useful for any disease state for which a botanical drug is associated. See for example Leung and Foster, 1996 and Herbal Drugs and Phytopharmaceuticals, 1994. More specific examples of disease states or therapeutic indications include AIDS, adaptogen, mild-to-moderate depression, anti-arthritic, anti-cancer, anti-diarrhetic, anti-helmenthic, anti-inflammatory, anti-nausea via GI, anti-rheumatic, anti-spasmodic, anti-ulcer, angina, antibacterial, antimutagenic, antioxidant, antiviral, arteriosclerosis, arthritis, asthma, blood pressure, benign prostatic hyperplasty (BPH), bronchial asthma, bronchitis, calmative, cough, cerebral circulatory disturbances, cholesterol lowering, cirrhosis, dermatological anti-inflammatory, diabetes, diuretic, drastic cathartic, dysmenorrhea, dyspepsia, emphysema, environmental stress, expectorant, free radical scavenger, GI distress, hemorrhoids, hepatitis, hepatoprotective, hypertension, hyperlipidemia, hyperprolactinemia, immunomodulatory activity, increase fibrinolysis, resistance to bacterial infection, inflammation, insomnia, lactation, liver protection, longevity, menstrual cycle regulation, migraine, muscle pain, osteoarthritis, pain, peripheral vascular disease, platelet aggregation, PMS, promote menstrual flow, prostatic disorders, reduce triglycerides, relieve menstrual pain, respiratory tract infections (RTI), retinopathy, sinusitus, rheumatism, sedative, sleep-promoting agent, sore throat, stimulate hair growth, superficial wound healing, tinnitus, topical eczema (dermatitis), urinary tract infection (UTI), varicose veins, venous insufficiency or wound healing.

Other indications include anti-hemorrhagic, anti-microbial, anti-parasitic, anti-pyretic, cardiotonic, carminitive, cholagogue, demulcent, diaphoretic, emetic, emmenagogue, emollient, febrifuge, galactagogue, hepatic, hypnotic, laxative, nervine, pectoral, rubefacient, stimulant, tonic, vulnerary, canker stores, pyorrhea, gingivitis, gastritis, ulcers, gallstones, intermittent claudication, cold, influenza, laryngitis, headache, shingles, cystitis, kidney stones, atopic vaginitis, uterine fibroids, osteoporosis and gout.

5.8. PHARMAPRINT® OF ECHINACEA

5.8.1. BIOLOGICAL PHARMAPRINT®

Exemplary biological PharmaPrint® values. derived using the methods described herein. are shown in Table 1 and 2. See. *infra*, Section 6.4 for a detailed discussion and explanation of each of the biological assays for each of the values presented in Table 1 and 2.

Values for each bioassay in Table 1 are expressed as a percentage range of inhibition at 10^{-4}M unless otherwise indicated. Calculations for extracts and fractions are based on an assumption of an average molecular weight of 200.

TABLE 1. BIOLOGICAL PHARMAPRINT VALUES

Extracts	TK	TNF-α*	PK P59	D4	EGF TC	PE	15-Lipo
Broad range	20±10	30±20	40±20	50±40	20±10	30±15	30±15
Narrow range	20±10	25±10	40±20	50±20	15±5	30±15	30±15
Fractions							
EP Frac. 1	35±15						
EP Frac. 2	30±15						
EP Frac. 3	25±10						
EP Frac. 5	25±10	——					
EP Frac.11	65±20						
EP Frac.12	55±20						
EP Frac.13	75±20						
EP Frac.14	55±20						
EP Frac.15	65±20						
EP Frac.16	60±20						
EP Frac.17	25±10						
EP Frac.18	65±20						
Reference Compounds							
Echinoside	45±20						
Cicoric acid	55±20						
Caffeic acid	35±20						

* TK, tyrosine kinase; TNFα, inhibition of TNFα binding to its receptor a 10^{-8} M; PK P59, protein kinase, 59 fyn; D4. leukotriene, D4; EGF, protein kinase, EGFTK, epidermal growth factor tyrosine kinase assay, PE, Phorbol ester, 15-Lipo, 15-lipoxygenase assay.

TABLE 2. BIOLOGICAL PHARMAPRINT

Pharmaprint Range of Actives

Bioassay Marker	Ranges (IC_{50})		
	Broad Range (Mean ± 3STD)	Medium Range (Mean ± 2 STD)	Preferred Range (Mean ± 1 STD)
15-Lipoxygenase (μM)	4.0 - 2400	20.0 - 1200	100 - 600
5-Lipoxygenase (μM)	25.0 - 600	120 - 400	210 - 330
Macrophage Activation Secretion of TNFα (pg/mL)	11.0 - 575	105 - 481	199 - 387
Tyrosine Kinase p59fyn (μM)	1.0 - 500	5.0 - 300	54.0 - 214
Protein Kinase p55fyn (μg/mL)	1.5 - 800	8.0 - 400	40.0 - 200

By way of example, using the values in Table 1 or 2, the PharmaPrint® is based on the bioactivity of extract in the inhibition of TNFα binding to its receptor and one or more assays selected from the following group: 15-lipoxygenase, 5-lipoxygenase, tyrosine assay p59fyn; protein kinase 55 fyn; leukotriene D4; protein kinase, EGF-TK, epidermal growth factor tyrosine kinase assay, phorbol ester assay, 15-lipoxygenase assay.

In an alternative embodiment, the PharmaPrint® may be developed based on bioactivity equal to or greater than the lower end of the range of bioactivity values such as shown in Table 1. As an illustrative example of this embodiment, the PharmaPrint® value based on the bioactivity of total extract in the leukotriene D4 assay (50± 20) would be at least 30% inhibition on at 10^{-4}M.

5.8.2. CHEMICAL COMPONENTS

A review of the relevant literature relating to echinacea reveals marker compounds illustrated below in Table 3. See, *infra*, Section 6.5 for a detailed discussion and explanation of the selection of the chemical compounds. An exemplary chemical PharmaPrint is shown in Table 4.

TABLE 3.

Range	Broad (% w/w)	Intermediate (% w/w)	Narrow (% w/w)
Caffeoyltartaric0	0-2.0	0-1.1	0.05-0.6
Cichoric	0.01-2.6	0.02-1.4	0.1-1.0
Echinoside	0-2.0	0-1.0	0.01-0.6
Total Phenol	0.01-3.1	0.02-2.3	0.06-1.8

TABLE 4. CHEMICAL PHARMAPRINT

Pharmaprint Range of *Echinacea purpurea*

Chemical Marker	Ranges (%) w/w		
	Broad Range (Mean ± 3STD)	Medium Range (Mean ± 2 STD)	Preferred Range (Mean ± 1 STD)
Total Phenols	1.17 - 5.47%	2.14 - 4.81%	2.80 - 4.14%
Cichoric Acid	0.90 - 4.81%	0.80 - 4.12%	1.38 - 3.43%
Isobutyl Amides	0.005 - 0.180%	0.01 - 0.114%	0.02 - 0.084%

5.8.3. CONVERSION RATIO

PharmaPrint® values developed using dry powdered extracts of a botanical material can be converted to values relevant to dry weight of raw botanical material using the ratios illustrated in Table 5 below. Thus, to convert PharmaPrint® values based on a dry powdered extract to values relevant to a dried plant material, one would divide by the appropriate factor in Table 5.

TABLE 5. CONVERSION RATIOS

CONVERSION RATIOS	
Botanical	**Ratio (powder to extract)**
Saw Palmetto	10:1
St. John's wort	5:1
Valerian	5:1
Echinacea	5:1
Ginkgo	50:1
Ginseng	5:1
V. agnus-castus	10:1
Black Cohosh	1:1
Bilberry	100.1
Milk Thistle	40:10

The following example is presented for purposes of illustration only and is not intended to limit the scope of the invention in any way.

6. EXAMPLE: *Echinacea angustifolia* DC, *E. pallida* (Nutt.) Nutt., and *E. purpurea* (L.) Moench.

6.1. COMMERCIAL SUPPLIERS/PRODUCT NAMES

Echinacea is one of the most popular botanical products available. One of the major proprietary products is Echinacin™, by Madaus AG (Koln, Germany), and a similar product is sold in the U.S. as Echinaguard™, by Murdock Madaus Schwabe (Springville, Utah).

Also, a powdered extract of *Echinacea angustifolia* is available (min. 4-5% echinosides) from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany). Other suppliers include Trout Lake Farm, PhytoPharmica, Herbal Choice-Boatalia, Shaklee, Botalia Gold, Nature's Herbs, Nature's Way, Flora Laboratories, and Herb Pharm.

6.2. FRACTIONAL ANALYSIS

The fractional analysis is performed using Sephadex™ chromatography. Alternatively, reverse phase C-18 chromatography or GPC chromatography may also be used. There are two major categories of bioactive components in echinacea, the non-polar material that is extracted with chloroform and the polar component which is in the ethanol or aqueous fraction. The non-polar lipophilic constituents include various anions as

described by Bauer and Foster (Bauer and Foster, 1991, *Planta Med.* 57:447-449). The polar components include the alkylamides and the polysaccharides (Bauer and Remiger, 1989, *Planta Med.* 55:367-371; Steinmuller et al., 1993, *J. Immunopharmacol.* 15(5):605-614).

Phytochemical investigation of *Echinacea sp.*

1. Fractionation of *Echinacea* extract

An extract prepared from *Echinacea sp.* Moench. (manufacturer Finzelberg, batch 6644270) was separated by chromatography on Sephadex LH-20. The resulting fractions were submitted for bioassay.

Separation conditions:

10 g of the extract was dissolved in 50% aq. ethanol and separated on 200 g Sephadex LH-20 with 50% aq. methanol as the mobile phase. 25ml fractions were collected, analyzed by HPLC and combined according to the HPLC chromatograms to yield 14 fractions. Methanol was removed in an air stream (Turbo-Vap. 40°C) and finally the fractions were freeze-dried.

HPLC parameters

column:	RP-18			
mobile phase:	A=0.05% Trifluoracetic acid B=Acetonitrile			
gradient:	t[min]	%A	%B	
	0	100	0	
	30	0	100	
	35	0	100	
flow rate:	1 ml/min			
detection:	UV 250 nm and light scattering detection			
reference compounds:	echinacoside			

Results:

fraction	tubes	total yield [mg]	test sample [mg]	comment
1.	1-2	1	0.9	
2.	3-5	1196	20.1	
3.	6-7	1653	20.7	
4.	8-9	2544	20.6	
5.	10-11	1140	20.3	
6.	12-13	654	20.8	
7.	14-16	865	20.4	
8.	17-18	148	20.8	contains echinacoside
9.	19-20	102	20.5	contains echinacoside
10.	21-22	84	20.4	contains echinacoside
11.	23	26	20.4	
12.	24-25	25	20.0	
13.	26-27	16	10.1	
14.	28-30	3	1.2	
15.	31-32	18	13.2	

2. Isolation of alkylamide containing fractions from *Echinacea angustifolia* DC.

Alkylamide containing fractions were isolated from roots of *Echinacea angustifolia* (literature: R. Bauer. H. Wagner. *Echinacea Handbuch für Ärzte. Apotheker und andere.* Stuttgart: Wiss. Verl.-Ges., 1990).

Plant material:

Echinacea angustifolia. radix (Alfred Galke GmbH, Gittelde. Germany)

Extraction:

The plant material (1000 g) was extracted with petrol/methyl *tert*-butyl ether (50:50) at room temperature. The extract was evaporated to dryness to yield 7.3 g.

Chromatography:

7.3 g of the extract was separated by medium pressure chromatography on silica.

column: 460 x 30

sample: 7.3 g dissolved in 15 ml petroleum ether/ethyl acetate

gradient: t[min]	petroleum ether	ethyl acetate	methanol
0	80	20	0
45	0	100	0
50	0	100	0
60	0	50	50
61	0	0	100

flow rate: 30 ml/min

fractions: 20 ml

Fractions were combined according to TLC results (petrol/ethyl acetate 80:20). All fractions were further investigated by H-NMR spectroscopy. Alkylamides typical for *Echinacea sp.* show diagnostic signal patterns in H-NMR spectra (olefinic signals between 5.2 and 7.6 and signals the butylamine moiety between 0.85 and 1.00 ppm). The spectra of the following fractions are similar, showing mixtures of different alkylamides.

Results:

fraction	tubes	total yield [mg]	test sample [mg}
12208-3-1	11-12	1066	22.9
12208-3-2	13-14	564	21.2
12208-3-3	15-17	305	25.3

6.3. BIOLOGICAL ACTIVITY ANALYSIS

Echinacea is used for the prophylaxis and treatment of mild to moderately severe colds, influenza and septic processes, topically for treating wounds and inflamed skin conditions. Its action is supposedly due to enhancement of the body's defenses by non-specific immunostimulation affecting primarily the phagocytic immune system.

6.3.1. MODULATION OF [125I] LABELED TUMOR NECROSIS FACTOR-α TO ITS RECEPTOR

The bioactivity of the echinacea total extract and fractions are analyzed in an assay which measures the modulation of the binding of [125I]tumor necrosis factor-α (TNF-α) to human TNF-α receptors. The receptors are partially purified from U-937 (human histiocytic lymphoma) cells in a modified Tris HCl buffer (pH 8.6) using standard techniques. A 200 g "receptor" aliquot is incubated with 62 pM [125I]TNF-α for 3 hours at 4°C. Non-specific binding is estimated in the presence of 50 nM TNF-α. The reaction is filtered through glass filters and washed 3 times to remove unbound ligand. The filters are counted in a liquid scintillation counter to determine [125I]TNF-α specifically bound to its receptor. Compounds are initially screened at a 10 μM concentration (Maloff and Delmendo, 1991, *Agents and Actions* 34:32-34). Assay reference data and literature compounds are listed below:

Assay Reference Data:

K^d	37 pM
B^{max}:	11 pMoles mg protein
Specific Binding:	65%

Compound	IC_{50} (nM)	Ki (nM)	nH
Interleukin-1α (IL-1α)	>500	-	-
*TNF-α	0.084	0.032	1.2
TNF-β	0.714	0.27	1.0

*Indicates standard reference agent used.

Five aqueous alcoholic extracts, 18 fractions and four reference standards were bioassayed in the TNF-α binding assay to determine how well they antagonize TNF-α binding to its receptor. Results are shown in Table 6A.

6.3.2. INHIBITION OF PRE-INFLAMMATORY ENZYMATIC ACTIVITY

Another clinical indication of echinacea is its anti-inflammatory activity. The anti-inflammatory activity may be analyzed using a variety of methods. Three *in vitro* assays frequently used include the cyclooxygenase-1, cyclooxygenase-2 and lipoxygenase assay. These assays are described below.

6.3.3. 5-LIPOXYGENASE ASSAY

5-Lipoxygenase catalyzes the oxidative metabolism of arachidonic acid to 5-hydroxyeicosatetraenoic acid (5-HETE), the initial reaction in the biosynthetic pathway leading to the formation of the leukotrienes. The procedure was as follows. 5-lipoxygenase assays were run using a crude enzyme preparation from rat basophilic leukemia cells (RBL-1). Test compounds were pre-incubated with the enzyme for 5 minutes at room temperature and the reaction was initiated by addition of substrate (arachidonic acid). Following an 8 minute incubation at room temperature, the reaction was terminated by addition of citric acid, and levels of 5-HETE were determined by 5-HETE radioimmunoassay (RIA). Compounds are screened at 30 μM (Shimuzu et al., 1984, *Proc. Natl. Acad. Sci. USA* 81:689-693).

The following reference compounds were used for the inhibition of 5-lipoxygenase: reference compounds, (IC_{50} (μM)): BW-755C, (6.6); nordihydroguaiaretic acid (NDGA), (0.26); phenidone, (30).

Compounds and fractins were screened at an initial concentration of 3×10^{-5}. If an activity of greater than 50% inhibition was observed at 3×10^{-5}, a full dose response curve was carried out.

6.3.4. CYCLOOXYGENASE-1 ASSAY

Cyclooxygenase-1 (from ram seminal vesicles), 125 units per assay tube, was pre-incubated with 1 mM GSH, 1 mM hydroquinone, 1.25 mM hemoglobin and test compound for 1 minute at 25°C. The reaction was initiated by addition of arachidonic acid (100 mM) and terminated after 20 minutes incubation at 37°C by addition of trichloroacetic acid (TCA). Following centrifugal separation and addition of thiobarbiturate, cyclooxygenase activity was determined by reading absorbance at 530 nm (Evans et al., 1987, *Biochem. Pharamac.* 36:2035-2037; Boopathy and Balasubramanian, 1988, *J. Biochem.* 239:371-377).

The following reference compounds were used for the inhibition of cyclooxygenase 1: reference compounds, (IC_{50} (μM)); aspirin, (240); indomethacin, (1.7).

Compounds and fractions were screened at an initial concentration of 3 X 10^{-4}. If an activity of greater than 50% inhibition was observed at 3 X 10^{-4}, a full dose response curve was carried out.

6.3.5. CYCLOOXYGENASE-2 ASSAY

Cyclooxygenase-2 (from sheep placenta), 80 units per assay tube, was pre-incubated with 1 mM GSH, 1 mM hydroquinone, 1.25 mM hemoglobin and test compound for 1 minute at 25°C. The reaction is initiated by addition of arachidonic acid (100 mM) and terminated after 20 minutes incubation at 37°C by addition of TCA. Following centrifugal separation and addition of thiobarbiturate, cyclooxygenase activity is determined by reading absorbance at 530 nm (Boopathy and Balasubramanian, 1988; Evans et al. 1987; O'Sullivan et al., 1992. *Biochem. Biophy. Res. Commun.* 187:1123-1127).

The following reference compounds were used for the inhibition of cyclooxygenase-2: reference compounds, (IC_{50} (μM)): aspirin, (660); indomethacin, (2.4).

Alternatively, production of arachidonate metabolites may also be analyzed following the procedure of (Wagner et al., 1989, *Planta Med.* 55:566-567). Standard *in vivo* assays such as the rat paw assay or the mouse ear assay are performed as described in the literature preparations (Tragni et al., 1985, *Chem. Toxic.* 23(2):317-319) or as described below.

6.3.6. MOUSE EAR DERMATITIS MODEL

Echinacea extract and fractions are administered i.p. (0.1, 1, 10 and 100 mg/kg) to groups of 5 ICR derived male mice weighing 22 ± 2 g one hour before sensitization to oxazolone (0.1 ml of 5% solution) applied to the preshaven abdominal surface. Seven days

later, animals are challenged with oxazolone (25 µl of a 2% solution) applied to the right ear, vehicle being applied to the left ear. After 24 hours, each mouse is sacrificed and ear thickness measured with a Dyer Model micrometer gauge. A 30 % or more (≥30) thickness change relative to the vehicle treated control group is considered significant and indicates possible immunostimulant activity. (Griswold et al., 1974, *Cell. Immunol.* 11: 198-204).

Compound	ED_{30} mg/kg i.p.
*Azimexone	100
Bestatin	1
Levamisole	30

*Indicates standard reference agent used.

6.3.7. THE RAT PAW SWELLING ASSAY

The paw inflammation experiment is performed as described below. Echinacea extract and fractions are administered p.o. (100 mg/kg) to a group of 3 Long Evans male or female overnight fasted rats weighing 150 ± 20 g one hour before right hind paw injection of carrageenan (0.1 ml of 1% suspension intraplantar). Reduction of hind paw edema by 30 % or more (≥30) three hours after carrageenan administration indicates significant acute anti-inflammatory activity. (Winter et al.. 1962, *Proc. Soc. Exper. Biol. Med.* 111: 544-547)).

Compound	ED_{30} mg/kg p.o.
Acetazolamide	>50
Aspirin	150
BW 755C	30
Clonidine	1
Diflunisal	30
Furosemide	50
Hydrocortisone	30
Hydroflumethiazide	>50
Ibuprofen	30
*Indomethacin	3
Ketoprofen	3
Naproxen	3
Phenidone	50
Phenylbutazone	50
Probenecid	>50
Salbutamol	10

*Indicates standard reference agent used

BW 755C=3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline.

6.3.8. TARGET CELL CYTOLYSIS

The extract is separated into its basic classes of components following the procedure set forth in FIG. 3. Each separated class of components (as well as the original extract) is subjected to two types of cytotoxicity bioassays. The first assay measures the activation of spleen cells in C57BL/6 mice. The mice are sensitized with P815Y lymphoma and treated with different doses of the sterile extract or specific components. After 11 days post lymphoma cell inoculation, the spleens are harvested and splenocytes are used to challenge P815Y lymphoma cells loaded with ^{51}Cr. The release of the radioactive ^{51}Cr from the tumor cells provides a quantitative measure (radioactive counts) of the activation of splenocytes (the assay system).

A second cytotoxicity assay measures the immuno- stimulatory properties of the extract and its fractions on macrophages. Adherent, thioglycolate induced, starch-induced or bone marrow derived macrophages have cytotoxic activity. Tumor cells labeled with ^{51}Cr (*e.g.*, P815 mastocytoma or WEHI164 fibrosarcoma cells) serve as the target cells. The isolated macrophages are seeded in flat bottom sterile micro- titer plates in RPMI1640-medium with 5% fetal calf serum. Sterile compounds are added and incubated for 24 hours at 37°C. After 24 hours. $2x10^4$ ^{51}Cr-labeled tumor cells are added to give a 10:1 effector-to target cell ratio. After an additional 24 hour incubation at 37°C the supernatants and sediments are separated and the radioactivity of both fractions is measured in a gamma counter.

The percent ^{51}Cr release is calculated as follows:

(Counts per minute in supernatant/counts per minute in supernatant + counts per minute in sediment) x 100. Specific ^{51}Cr release is the difference in the ^{51}Cr release between target cells cultured in the presence and absence of the substance using control or activated macrophages (Stimpel, M. et al. *Infection and Immunity* 46:845-849, 1984). Positive literature compounds for this assay are γ-interferon and lipopolysaccharide (LPS).

6.3.9. BIOASSAY FOR MACROPHAGE ACTIVATION

Echinacea extract and its polysaccharides can stimulate macrophage activity. In experimental systems, macrophages can be obtained by i.p. injection of mice with 2-3 ml. of 2% starch or thioglycolate. After 4-5 days peritoneal cells of the mice are collected with i.p. injection of 5 ml. PBS. The buffer is removed with a syringe and the harvested cells are collected by centrifugation and incubated at 37°C in RPMI 1640 medium containing 10% fetal calf serum. After one (1) hour, non-adherent cells are discarded and the remainder used for testing. The macrophages are treated with different concentrations of echinacea

extract and the echinacea fractions in the culture media and activation qualified by the cytotoxicity against ^{51}Cr loaded P815 cells as targets (Stimpel et al. 1984, *Infection and Immunity* 46:845-849). A second method to measure macrophages activation involves the culture of the mouse macrophage cell line MH-S(Mbawiuke, I.N. and Herscowitz J. of *Leukocyte Biol* 46:119-127, 1989). These cells grow as adherent cells in RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum. The MH-S cells are plated in a 96-well sterile flat-bottom micro-titer plate and incubated 18 hours with compounds. Culture supernatants are removed and secreted TNF-α is measured in a biological assay using dactinomycin treated L929 cells. The measured units are the reciprocal values of the supernatant dilution that would cause lysis of 50% of the L929 cell layer (Luetting, B. et al. *J Natl. Cancer Inst.* 81:669-675, 1989). Literature reference compounds include LPS, γ-interferon and the polysaccharide arabinogalactan (Sigma Chemical Company).

Nine additional bioassays were performed on either extracts, fractions or reference compounds for Echinacea. Four of these assays focused on the inhibitory properties of the substances for a variety of tyrosine kinases. The first tyrosine kinsase assayed was the epidermal growth factor tyrosine kinase (EGF TK). Basically this assayed involved taking a cDNA encoding the intracellular tyrosine kinase domain of the human EGF receptor (EGF-TK) and expressing it in a baculovirus expression system in Sf9 insect cells. The kinase assay measures activity of the 69 kD kinase domain by employing an immobilized synthetic polypeptide as substrate. Following a 10 minute reaction the phosphorylated tyrosine residues are detected by incubation with a monoclonal anti-phosphotyrosine antibody. Bound anti-phosphotyrosine antibody is quantitated by incubation with a biotin-linked anti-mouse IgG, followed by streptavadin-linked β-galactosidase enzyme. Fluorescence resulting from conversion of fluorescein-di-β-galactoside to fluorescein is measured. The reaction with fluorescein-di-β-galactoside is stopped by addition of phenylethyl-β-D-thiogalactoside, a reversible competitive inhibitor of β-galactosidase (Geissler, J.F. *et at. J. Biol. Chem.* **265**: 22255-22261, 1990). The second kinase tested was the $p59^{fyn}$ tyrosine kinase (FYN TK) partially purified from bovine thymus. A fluorescent end-point ELISA employing an immobilized synthetic polypeptide as a substrate is used as the substrate. Substance and/or vehicle is pre-incubated with the enzyme for 15 minutes. Following a 10-minute kinase reaction in the presence of 100 μM ATP, phosphorylated tyrosine residues are detected as described for the EGF TK (Appleby, M.W. et al. *Cell* **70**: 751-763, 1992). The third tyrosine kinase assay measures the enzyme activity of $p56^{lck}$ tyrosine kinase (LCK

TK) partially purified from bovine thymus. The fluorescent end-point ELISA employs an immobilized synthetic polypeptide as a substrate. Substance and/or vehicle is pre-incubated with the enzyme for 15 minutes. Following a 10 minute kinase reaction in the presence of 100 μM ATP, phosphorylated tyrosine residues are detected by incubation with an anti-phosphotyrosine antibody as previously described (Hatakeyama, M. et al. Science *252*: 1523-1528, 1991). The fourth kinase (Syn TK) tested also uses a non-radioactive ELISA based method. A defined biotinylated peptide sequence is first added to NeutrAvidin coated wells where it binds. Addition of cellular tyrosine kinases results in the phosphorylation of tyrosine residue(s) within the peptide. The anti-phosphotyrosine antibody, PY20, conjugated with horseradish peroxidase (HRP) is added to specifically detect phosphotyrosine residues. The tyrosine kinase activity is then determined by addition of a soluble substrate to the well. Briefly, 50 μl of diluted biotinylated tyrosine peptide (10 μg/ml) is dispensed into all required wells of a NeutrAvidin coated ELISA plate (Pierce, Rockford, IL). The plates are covered and incubated for 30 minutes at 37°C. The plates are washed 3X with 300 μl wash buffer (100 mM Tris, 150 mM NaCl, 0.005% Tween 20, pH 7.5). Each wash has a two minute pause/incubation. Any remaining fluid following the final aspiration is removed by striking the plates in absorbent toweling a minimum of three times. The substances are tested in working reaction buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 5mM dithiothreitol, 1mM $NaNO_4$, 2 mM ATP, 20 mM $MgCl_2$, 1 mM AEBSF 4-(2-aminoethyl)benzenessulfonyfloride), 1 μg/ml TLCK (Nα-p-tosyl-$_L$-lysine chloromethyl ketone), 0.5. μg/ml leupeptin, 0.5 μg/ml soybean tyrosine kinase peptide, and incubated for 30 minutes at 37°C. The plates were then washed via the wash procedure described above and incubated with horseradish peroxidase conjugated monoclonal anti-phosphotyrosine antibody for one hour at 37° C. The wash procedure is then performed followed by the addition of 100 μL TMB substrate. Plates are then read at O.D. 650 after fifteen minutes incubation at 20°C (personal communication Dr. Joe Ringer, Paracelsian, Ithaca, NY).

Potential anti-inflammatory activities of the substances were tested in the following bioassays. First, inhibition of leukotriene C_4 synthetase was assayed using a partially purified receptor from rat basophilic leukemia cells (RB-1). The assay reactions are carried out using LTA_4 methyl ester as the substrate. The reactions are done in the presence of albumin, serine borate (prevents conversion of LTC_4 to LYD_4) and the substances. The reactions are run for 15 minutes at 25°C and are quenched by the addition of ice-cold methanol. The formation of LTC_4 is determined by RIA (Bach, M.K. et al. *Biochem. Pharmacol.* **34**: 2695-2704, 1985). Another assay investigating the potential anti-

inflammatory properties of the substances involves investigations with the enzyme 5-lipoxygenase (5-lipoxygenase). Crude enzyme is prepared from rat basophilic leukemia cells (RB-1). The substances are pre-incubated with the crude enzyme preparation for five minutes at 25°C. The reaction is then initiated by addition of [^{14}C] arachidonic acid. Eight minutes later the reaction is terminated by the addition of citric acid. The amount of radiolabeled 5-HETE is determined by radioimmunoassay (RIA) (Shimuzu, T. et al. *Proc. Natl. Acad. Sci. USA* **81**: 689-693, 1984). Inhibition of 15-Lipoxygenase (15 Lipoxygenase) was also tested. A partially purified enzyme was made from soybean and was incubated with substance and/or vehicle for 4 minutes at 25°C. The reaction is then initiated by addition of linoleic acid as substrate. Following a 4-minute incubation at 25°C the reaction is terminated by addition of NaOH. The amount of 15-HETE produced is determined by measuring its absorbance at 234 nm (Kemal, C., et al. *Biochemistry* **26**: 7064-7072, 1987). The last assay tested with potential anti-inflammatory activities was leukotriene C_4 (LTC_4) synthetase (LTC_4) and is assayed using a crude enzyme preparation made from rat basophilic leukemia cells (RB-1). A methyl ester of LTC_4 is incubated with the crude enzyme preparation in the presence of albumin and serine borate for 15 minutes at 15°C. The reaction is terminated by the addition of ice-cold methanol. Formation of LTC_4 is taken as an index of enzyme activity using an RIA readout method (Bach et al. *Biochem. Pharmacol.* **34**: 2695-2704, 1985).

The final assay tested for activity in the substances was the binding of [^{3}H]PDBu to phorbol ester receptors partially purified from whole brain membranes of male ICR derived mice weighing 20 ± 2 g. The assay reactions are carried out in a modified Tris-HCl (pH 7.5) buffer using standard techniques. A 20 µg aliquot of receptors is incubated with 3 nM [^{3}H]PDBu for 60 minutes at 25°C. Non-specific binding is estimated in the presence of 1 µM PDBu. Membranes are filtered and washed 3 times and the filters are counted to determine [^{3}H]PDBu specifically bound (Ashendel, C.L. *Biochem. Biophys. Acta* **822**: 219-242, 1985).

6.3.10. ANTIBIOTIC ACTIVITY

A bioassay for a secondary clinical indication is the antibacterial activity associated with the caffeic acid derivatives of the extract. The assay follows standard bacterial testing evaluating a panel of common pathogenic bacteria routinely assayed in quality control

testing laboratories or in clinical laboratories. Several manufacturers have commercial laboratories that routinely perform these assays.

The five extracts were also tested in an antimicrobial assay measuring the minimal inhibitory concentration (MIC) for *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Candida albicans* (see summary table 3 below). The first extract, EP101, is echinacea root from Finzelberg Botanicals International (Long Beach, CA). The second extract is echinacea dry extract (EP102) from Indena (Seattle, WA) and echinacea extracts EP 104 and 105 are dry extracts from Euromed, USA (Batch 32097 and 35896 respectfully, Pittsburgh, PA).

Fractionation using Sephadex LH-20 of an extract resulted in 18 samples and were generated by Analyticon (Berlin, Germany). The reference chlorogenic acid was from Sigma (St. Louis, MO), while echinoside, alkylamides and chicoric acid were from Analyticon (Berlin, Germany). The activity of the extracts is shown in Table 6 and 7 below.

Table 6A

Extracts	% Inhibition				
	TNF-α (@10^{-8}M)	MMIC	EGF TK @ 100µM	FYN TK @ 100µM	Leukotriene D_4 @ 100µM
EP104	26*	-	NT	NT	NT
EP101	30*	-	51	75	22
EP102	28*	-	21	20	-
EP103	32*	-	31	25	-
EP104	-	-	-	-	NT
EP105	-	-	-	-	NT
EP107	-	-	-	-	NT

TABLE 6B

Extracts	% Inhibition				
	Phorbol Ester @ 100µM	LCK TK @ 100µM	15-Lipoxygenase	Leukotriene C_4 Synthetase @ 100µM	5-Lipoxygenase @ 100µM
EP100	NT	NT	NT	NT	NT
EP101	27	23	28	28	-
EP102	30	-	-	-	-
EP103	-	-	25	-	31
EP104	NT	NT	NT	NT	NT
EP105	NT	NT	NT	NT	NT
EP107	NT	NT	NT	NT	NT

TABLE 7

Extracts	% Inhibition SYN TK @ 100µM
EP101	24
EP103	-
EP103	-
EP107	27
FRACTIONS	
Echinacea Fraction 1	36
Echinacea Fraction 2	27
Echinacea Fraction 3	23
Echinacea Fraction 4	-
Echinacea Fraction 5	23
Echinacea Fraction 6	-
Echinacea Fraction 7	-
Echinacea Fraction 8	-
Echinacea Fraction 9	-
Echinacea Fraction 10	-
Echinacea Fraction 11	63
Echinacea Fraction 12	-
Echinacea Fraction 13	74
Echinacea Fraction 14	55
Echinacea Fraction 15	65
Echinacea Fraction 16	58
Echinacea Fraction 17	24
Echinacea Fraction 18	67

Reference Compounds	% Inhibition
Chlorogenic acid	-
Echinoside	46
Alkamides	NT
Chicoric acid	53.5
Caffeic acid	32
Dienoic amides	-

6.3.11. ADDITIONAL INFLAMATORY ACTIVITY BIOASSAYS

Lipoxygenases are lipid-peroxidating enzymes implicated in the pathogenesis of a variety of inflammatory disorders. The 5-lipoxygenase enzyme is involved in the first step of the biosynthesis of such potent pro-inflammatory mediators such as the leukotrienes. 15-lipoxygenase is involved in the biosynthesis of other bioactive metabolites derived from free arachidonic acid. Interestingly, 15-lipoxygenase is unique among the human lipoxygenases in that it is able to oxygenate polyenoic fatty acids esterified to membrane lipids or lipoproteins and therefore may have biologic effects distinct from free arachidonic acid (Segal, E. and D.J. Conrad *Advances in Thromboxane and Leukotriene Research* 22: 309. 1994). In man, 15-lipoxygenase is expressed preferentially in the epithelial cells of the airway, and macrophages, suggesting a key role in inflammatory responses of the upper respiratory tract frequently seen in the common cold. Surprisingly, preferential expression of 15-lipoxygenase is also seen in atherosclerotic lesions suggesting a potential anti-atherosclerotic activity for *Echinacea.* To study the affects of the compositions on the enzymatic activity of 15-lipoxygenase the following assay was performed. Rabbit reticulocytes were used as the source of the enzyme (15 U reaction). The inhibition of the conversion of 256 μM linoleic acid (source of arachidonic acid) to 15-HETE was measured spectrophotometrically at 660 nm according to the manufacturers directions. The reaction was carried out at 4°C for 10 minutes in phosphate buffered saline (pH 7.4). At the conclusion of the reaction it was terminated by the addition of N-benzoyl leucomethylene blue (Auerbach, B. J. et al. *Anal. Biochem.* 201: *375,* 1992). The reference compound NDGA (Nordihydroguaiaretic acid had an IC_{50} (pM) 0.9.

$p55^{fyn}$ is a member of the src-related gene family of non-receptor and membrane associated tyrosine kinases. These kinases play an important role in mediating signal transduction through the T-cell receptor thereby initiating a signal transduction cascade leading to lymphokine secretion and cell proliferation. One of the earliest biochemical responses following T-cell receptor activation is an increase in tyrosine kinase activity and concomitant phosporylation of several intercellular substrates. In case of the B-cell receptor, fyn is one of several kinases associated with the receptor and is activated following receptor cross-linking. The tyrosine kinase is partially purified from bovine thymus. Phosphorylation is measured using a [^{33}P] poly GT peptide as the substrate and scintillation counting of the labeled protein as the end-point. The test compound is preincubated with the enzyme for 15 minutes and is then reacted for 10 minutes in the presence of 100 N M ATP (Cheng, H. C. et al. *J. Blot. Chem.* 267: 9248, 1992). The reference compound Staurosporine had an IC_{50} (μM) of 0.06.

To determine the bioactivity of the botanical *Echinacea* in stimulating the mouse macrophage cell line MH-S (Mbawuike and Herscowitz. J. Leuk. Riot. 46:119, 1989) to secrete TNF-α (Luettig et al.. J.Natl. Cancer Inst. 81:669, 1989) the following cell-based assay was performed. The MH-S cell line was obtained from ATCC (CRL-2019). The cells are grown in RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1 mM sodium pyruvate and supplemented with 0.05 mM 2-mercaptoethanol, 90%; and 10% fetal bovine serum (complete medium). The cells are subcultured using 0.25% trypsin and 0.03% EDTA solution, rinsed and removed. The cells are left at room temperature for approximately five minutes until the cells are detached. Detached cells are re-cultured in complete medium. The doubling time of this cell line is approximately 20 hours (where 3 X 10^7 cells per T175 flask are seeded). Care is taken to be sure the cells are in the exponential phase of growth and each passage is recorded for each assay. Secreted murine TNF-α is detected in the culture supernatant by ELISA (R&D Systems, catalog number MTA00, Minneapolis, MN). Cell viability is detected after incubation with the test compound using the MTT assay. Essentially this assay involves 8 X 10^4 cells are plated in 96-well sterile microtiter plates (Falcon) in 0.2 ml complete RPMI-1640 medium for six hours. Complete medium is removed and test compound in complete medium is added to duplicate wells as a two-fold dilution series in the range of 500 μg/mL to *7.5* μg/ml. The cells are incubated 18-20 hours at 37^{0}C, *5%* CO_2. The test medium is removed and reserved for determining the amount of secreted TNF-α. Fifty μl of the culture supernatant is used in duplicate for this analysis using the TNF-α ELISA kit (R&D Systems). The amount of TNF-α secreted is determined by comparison to the control TNF-α standard curve. The amount of secreted TNF-α is reported as pg/ml. The ELISA assay is carried out in a 96-well flat-bottom microtiter plate

(part of the assay kit) according to the manufacturers directions. Results of these inflammatory activity assays are shown in Tables 8 and 9.

TABLE 8.

Sample	5-Lipoxygenase Enzymatic activity (EC_{50}=mM)	15-Lipoxygenase Enzymatic activity (EC_{50}=mM)	p^{fyn} Tyrosine Kinase activity (EC_{50}=mM)	$p55^{fyn}$ Tyrosine Kinase activity (EC_{50}=mg/ml)	Leukotriene C4 Synthetase (EC_{50}=mg/ml)
Extracts (average)	0.271 ± 0.06	0.094 ± 0.044	0.134 ± 0.08	0.072 ± 0.03	NA
Fraction					
100% H_2O fraction	NA	NT	0.153	NT	NA
2.5%MeOH	NA	0.021	0.116	NT	NA
5% MeOH	NA	0.078	0.189	NT	NA
10%MeOH	17	0.101	0.217	NT	NA
20%MeOH	60	NA	0.104	NT	60%@0.1mM
40% MeOH	69	0.041	0.095	NT	69%@0.1mM
80%MeOH	85	0.099	0.123	NT	80%@0.1 mM
100% MeOH	37	0.291	0.2 13	NT	0.694
100% acetone	88	NT	0.313	NT	88%@0.1 mM
Compound					
Caffeic acid	0.0058	0.0012	NA	NA	NA
Chlorogenic acid	NA	0.0048	NA	NA	NA
Cichoric acid	NA	0.0018	NA	0.00299	NA
Coumaric acid	NT	NT	NT	NT	0.309
Echinacoside	NA	0.0061	NA	NA	NA

* Assumed a FW of 200. NT= not tested NA= not active

TABLE 9.

Sample	Macrophage Activation (Secretion of TNFα @ 500 µg/ml test concentration)
Echinacea purpurca EP 107 extract (ave)	293 ±94 pg/ml
Fraction	
1987-016-Fl	13±3pg/ml
1987-016-F2	19.5 ± 6.4 pg/ml
1987-016-F3	8 ± 11.3 pg/ml
1987-016-F4	1.5±2 pg/ml
1987-016-F5	NA
1987-016-F6	4.5 ± 6.4 pg/ml
1987-016-F7	2± 1.4 pg/ml
1987-016-FS	7 ± 3 pg/ml
1 987-016-F9	54.5 ± 7.8 pg/ml
1987-016-F10	33.5±pg/ml
1987-016-F11	25.5 ± 6.4 pg/ml
1987-016-F12	NA
1987-016-F13	0.5 ± 0.7 pg/ml
1987-016-F14	NA
1987-016-FIS	NA
2058-39-10 (Carbohydrate fraction)	61.5 ± 7.8 pg/ml
1991-180-18 (Carbohydrate fraction)	64.5±2.1 pg/ml
Sample Compound	
Caffeic acid	NT
Chlorogenic acid	4.5 ±2.1 pg/ml
Cichoric acid	8± 1.4 pg/ml
Coumaric acid	NT
Echinacoside	8.5 ± 2.1 pg/ml
Acidic arabinogalactan	4± 1.4 pg/ml
Isobuty l amides	NA

NA = not active; NT= not tested

6.4. CHEMICAL ANALYSIS HPLC, GPC

The most bioactivity associated components (*e.g.* terpenoids) are separated by GC, GC/MS or HPLC technology into individual components which are used to provide fingerprints in accordance with the present invention.

In hydroalcoholic preps the following compounds are found: echinacosides, arabinogalactan, heteroxylan (activity potentiated by isobutylamides, chicoric acid). The detailed analysis of the chemically-active components of Echinacea is performed using published procedures (Bauer and Foster, 1991; Bauer and Reminger, 1989; Bauer et al., 1989, *Zeit. fur Phytotherapie* 10:43-48). FIG. 4, FIG. 5 and FIG. 6 show examples of the chemical constituents found in analysis of commercially available echinacea samples.

Six (6) encapsulated echinacea samples were analyzed four were plant powder capsules and two were extracts. The determination of the concentration of total phenols in the samples was requested. The average capsule content weights were determined. Concentrations of caffeoyl-tartaric acid, chlorogenic acid, echinacoside, and cichoric acid were was determined by HPLC see descriptions above. Quantification for echinacoside was performed based on a two point standard curve for the response of an isolated echinacoside standard. Quantification of caffeoyl-tartaric acid and cichoric acid was performed based on a two point standard curve for a chlorogenic acid standard purchased from Sigma and the responses were adjusted using standard experimentally derived factors. Results are in Table 10 and FIG. 7.

The concentrations listed in Table 11 are based upon the average capsule content weights (shown in Table 9) and represent the average of two independent sample preparations for each sample. The total phenols reported are a sum of the individual phenols reported in the table.

TABLE 10. HPLC ANALYSIS IN WEIGHT/WEIGHT %.

Sample	A	B	C	D	E	F
Caffeoyltartaric	0.453	0.092	0.07	0.405	0.305	0.237
Chlorogenic	0.017	0.004	0.016	0.01	0.01	0.009
Echinoside	0.01	0.004	0.57	0	0	0
Cichoric	0.918	0.056	0.177	0.493	0.793	0.984
Total Phenols	1.398	0.156	0.833	0.908	1.108	1.23

TABLE 11. AVERAGE CAPSULE WEIGHTS:

Sample ID	Average Capsule Content wt.(mg)
A	410.8
B	748.9
C	441.8
D	385.3
E	415.9
F	452.4

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed since these embodiments are intended as illustration of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed. various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Throughout this application various publications and patents are cited in parenthesis. Their contents are hereby incorporated by reference into the present application.

We claim:

1. A method for determining whether echinacea is a pharmaceutical grade echinacea, the method comprising the steps of:

providing an echinacea material which has a given biological activity, said echinacea material comprising a plurality of components;

separating a representative aliquot of the echinacea material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active component;

determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the representative aliquot; and

comparing the bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade echinacea to determine whether the echinacea material is a pharmaceutical grade echinacea.

2. The method according to claim 1, wherein one or more of the marker fractions contain one active component.

3. The method according to claim 1, wherein the method comprises the additional steps of:

determining the amount of the active components in at least one of the marker fractions to provide a quantitative compositional fingerprint of the representative aliquot and

comparing the quantitative compositional fingerprint of the representative aliquot to a quantitative compositional fingerprint standard which has been established for a given pharmaceutical grade echinacea to determine whether the echinacea material is a pharmaceutical grade echinacea.

4. The method according to claim 1, wherein the method comprises the additional steps of:

determining a total bioactivity of the representative aliquot of the echinacea material and

comparing the total bioactivity of the representative aliquot with that of a total bioactivity of the standard to determine whether the echinacea material is a pharmaceutical grade echinacea.

5. The method according to claim 1, wherein the echinacea material is a supercritical carbon dioxide extract, an ethanolic extract, an aqueous or organic extract, a seed oil or a powdered plant material.

6. The method according to claim 1, wherein the echinacea material is a homogeneous material.

7. The method according to claim 1, wherein the echinacea material is a mixture of plant materials.

8. The method according to claim 1, wherein the active component is selected from the group consisting of cinnamic acid derivatives, polyketides, polyphenols, polysaccharides, proteins, prostaglandins, steroids and terpenoids.

9. The method according to claim 1, wherein the active component is selected from the group consisting of chlorogenic acids, echinosides, alkylamides, cichoric acids, caffeic acid and dienoic amides.

10. The method according to claim 1, wherein the active component is selected from the group consisting of alkylamides, echinosides, cicoric acid and caffeic acid.

11. The method according to claim 1, wherein the bioactivity is indicative of use for treating or ameliorating with an allergic/inflammatory disorder or a disease induced by a microbial organism or a virus.

12. A method for determining whether echinacea is a pharmaceutical grade echinacea, the method comprising the steps of:

providing an echinacea material which comprises a plurality of components which have a given biological activity and wherein each component has a standardized bioactivity profile;

separating a representative aliquot from the echinacea material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one of the active components;

measuring the amount of each of the active component(s) present in each of the marker fractions;

calculating the bioactivity of each of the marker fractions based on the amount of each of the active components present and the standardized component bioactivity profile to provide a calculated bioactivity fingerprint of the representative aliquot; and

comparing the calculated bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade echinacea to determine whether the echinacea material is a pharmaceutical grade echinacea.

13. The method according to claim 11, wherein the method comprises the additional steps of:

determining a total bioactivity of the representative aliquot of the echinacea material and

comparing the total bioactivity of the representative aliquot with that of a total bioactivity of the standard to determine whether the echinacea material is a pharmaceutical grade echinacea.

14. The method according to claim 13. wherein the echinacea material is an aqueous or organic extract.

15. The method according to claim 13. wherein the echinacea material is a powdered plant material.

16. The method according to claim 11. wherein the echinacea material is a homogeneous material.

17. The method according to claim 11. wherein the echinacea material is a mixture of plant materials.

18. The method according to claim 11. wherein the active component is selected from the group consisting of cinnamic acid derivatives. polyketides, polyphenols, polysaccharides, proteins, prostaglandins. steroids and terpenoids.

19. The method according to claim 11. wherein the active component is selected from the group consisting of chlorogenic acids. echinosides. alkylamides. chicoric acids. caffeic acids and dienoic amides.

20. The method according to claim 11, wherein the bioactivity is indicative of use for treating or ameliorating with an allergic/inflammatory or a disease induced by a microbial organism or a virus.

21. The method according to claim 1 or 11, wherein the marker fractions comprise a class of related components.

22. A method for determining whether echinacea is a pharmaceutical grade echinacea, the method comprising the steps of:

providing a echinacea material which has a given biological activity, said echinacea material comprising a plurality of components;

separating a representative aliquot of the echinacea material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active class of components;

determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the representative aliquot; and

comparing the bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade echinacea to determine whether the echinacea material is a pharmaceutical grade echinacea.

23. The method according to claim 22. wherein the active class of components are selected from the group consisting of cinnamic acid derivatives, polyketides, polyphenols, polysaccharides, proteins, prostaglandins, steroids and terpenoids.

24. A method for determining whether echinacea is a pharmaceutical grade echinacea which comprises determining a total bioactivity of a representative aliquot using at least two bioassays selected from the group consisting of tyrosine kinase assay (TK), TNF-α, EGF tyrosine kinase, $p59^{fyn}$ tyrosine kinase and leukotriene D4 and comparing the total bioactivity of the representative aliquot with that of a standard to determine whether the echinacea material is a pharmaceutical grade echinacea.

25. A method for making a pharmaceutical grade echinacea the method comprising the steps of:

separating a representative aliquot of a echinacea material, said echinacea material comprising a plurality of components, into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active component;

determining the amount of the active components in at least one of the marker fractions to provide a quantitative compositional fingerprint of the representative aliquot and

comparing the quantitative compositional fingerprint of the representative aliquot to a quantitative compositional fingerprint standard which has been established for a given pharmaceutical grade echinacea to determine whether the echinacea material is a pharmaceutical grade echinacea.

26. The method for determining whether echinacea is a pharmaceutical grade echinacea which method comprises:

determining a total bioactivity of a representative aliquot of an echinacea material and

comparing the total bioactivity of the representative aliquot with that of a total bioactivity of the standard to determine whether the echinacea material is a pharmaceutical grade echinacea.

27. A pharmaceutical grade echinacea obtained by the method according to claims 1, 11, 22. 24 or 25.

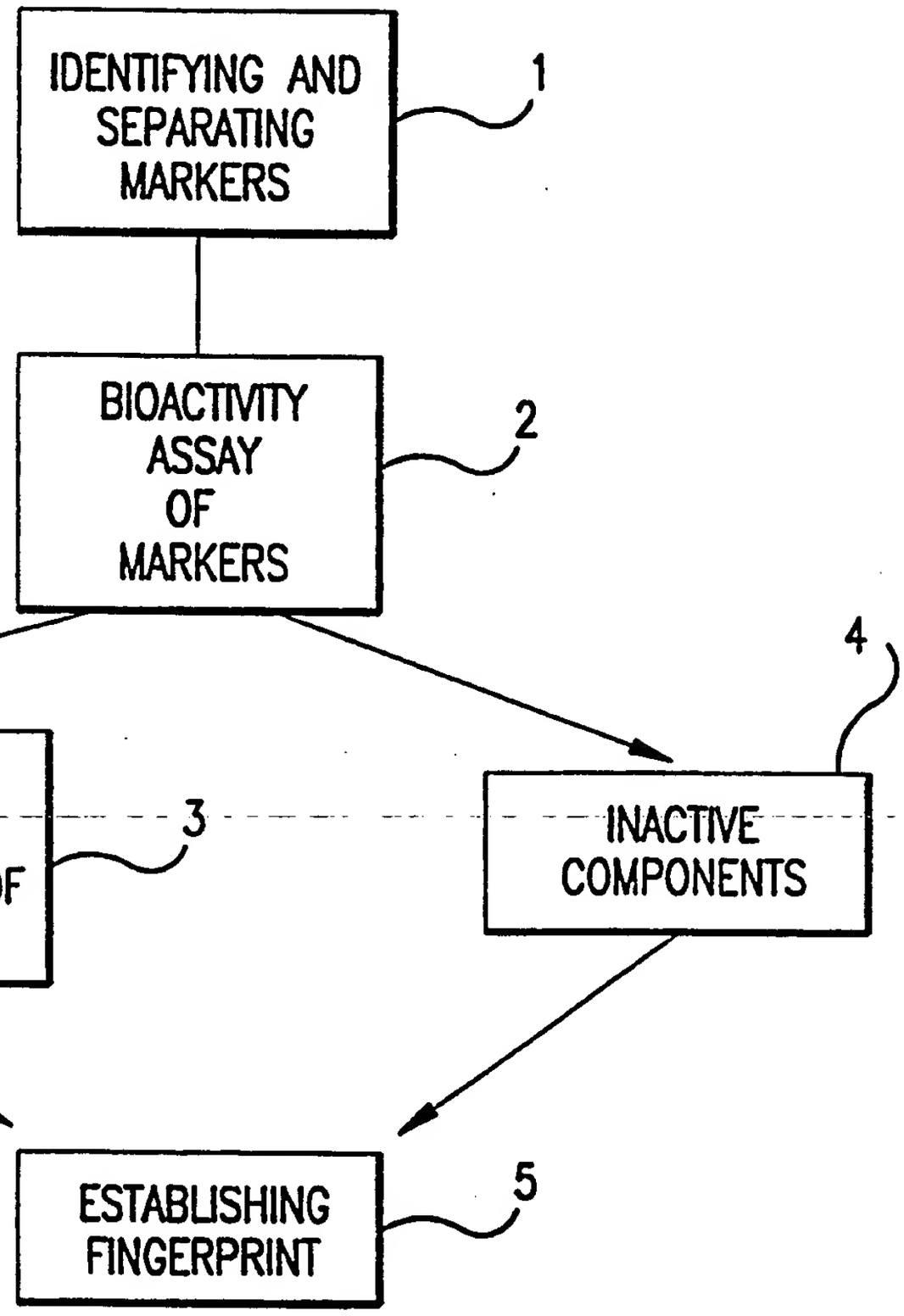

IDENTIFYING AND SEPARATING MARKERS
1
BIOACTIVITY ASSAY OF MARKERS
2
COMPONENTS HAVING A SUBSTANTIAL PORTION OF ACTIVITY
3
INACTIVE COMPONENTS
4
ESTABLISHING FINGERPRINT
5

FIG.1

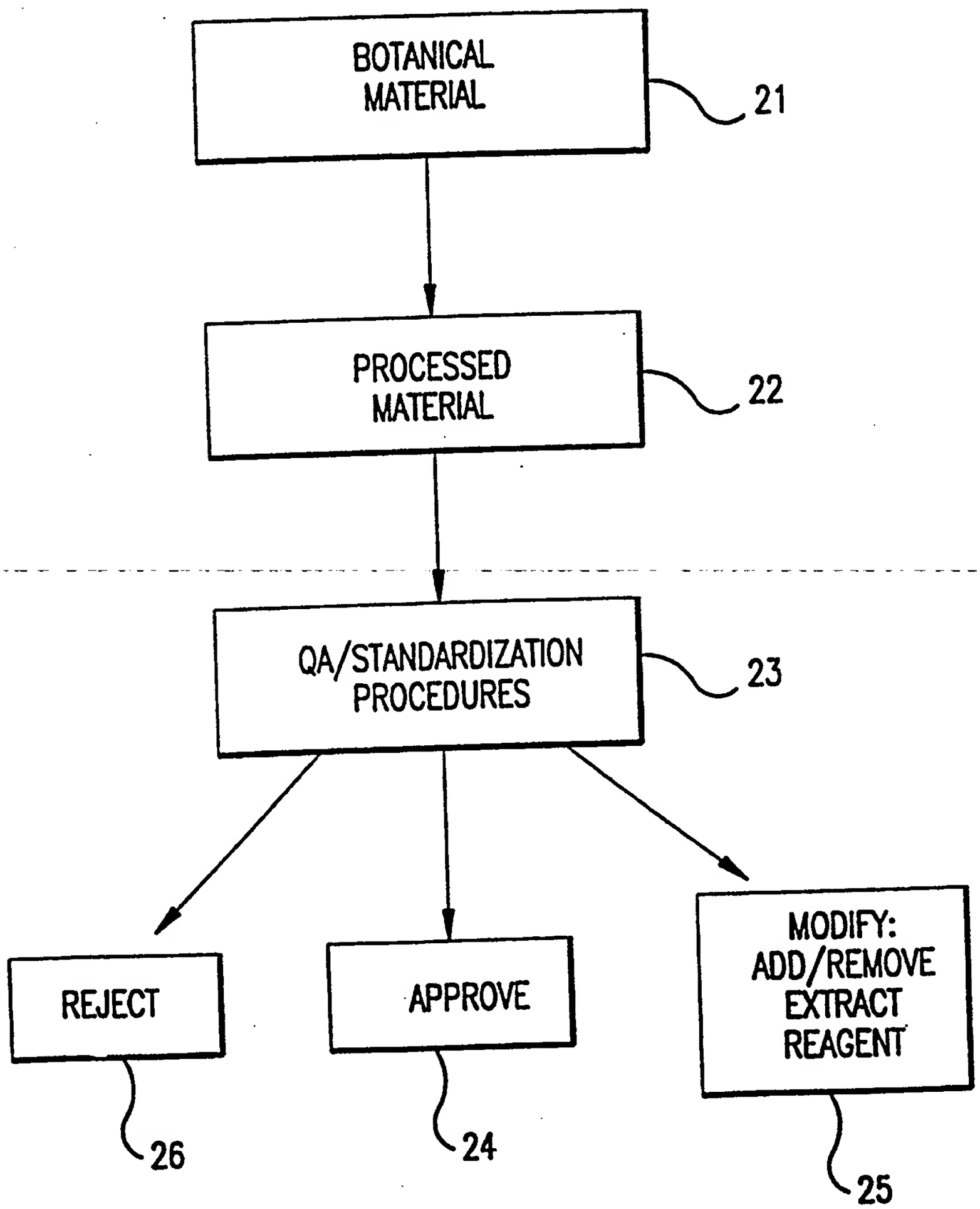

BOTANICAL MATERIAL
21
PROCESSED MATERIAL
22
QA/STANDARDIZATION PROCEDURES
23
REJECT
26
APPROVE
24
MODIFY: ADD/REMOVE EXTRACT REAGENT
25

FIG.2

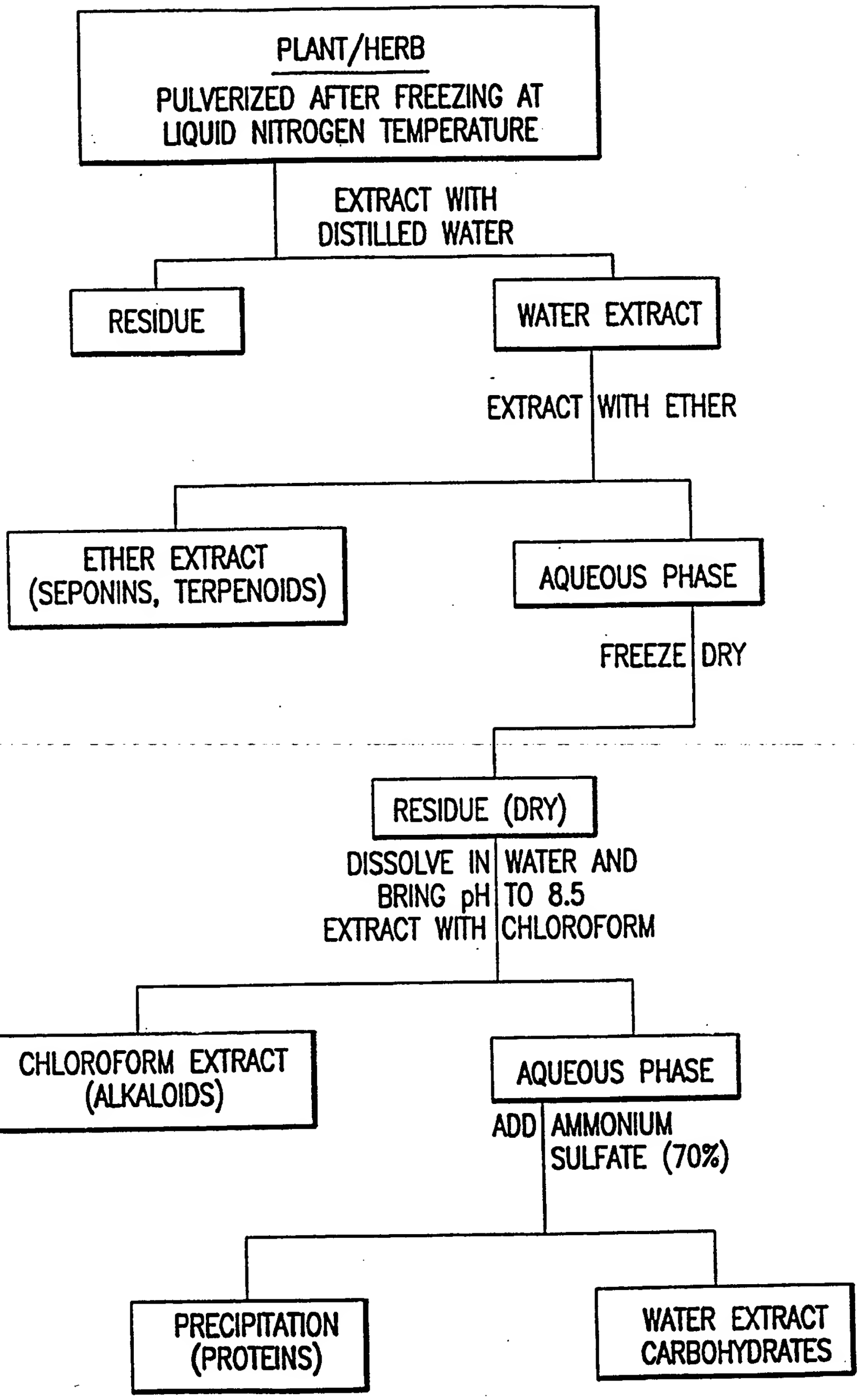

PLANT/HERB
PULVERIZED AFTER FREEZING AT LIQUID NITROGEN TEMPERATURE
EXTRACT WITH DISTILLED WATER
RESIDUE
WATER EXTRACT
EXTRACT WITH ETHER
ETHER EXTRACT (SEPONINS, TERPENOIDS)
AQUEOUS PHASE
FREEZE DRY
RESIDUE (DRY)
DISSOLVE IN WATER AND BRING pH TO 8.5 EXTRACT WITH CHLOROFORM
CHLOROFORM EXTRACT (ALKALOIDS)
AQUEOUS PHASE
ADD AMMONIUM SULFATE (70%)
PRECIPITATION (PROTEINS)
WATER EXTRACT CARBOHYDRATES

FIG.3

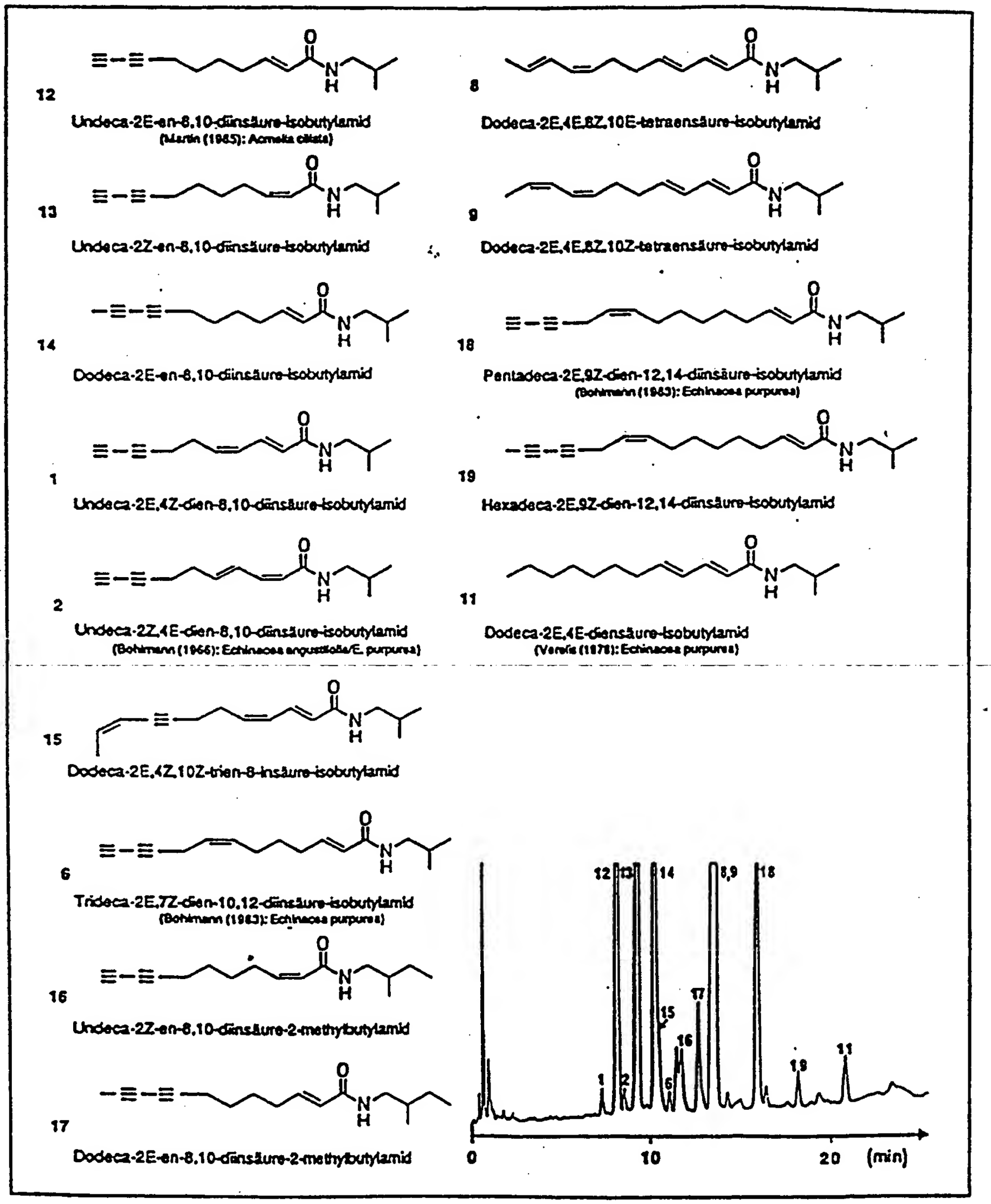
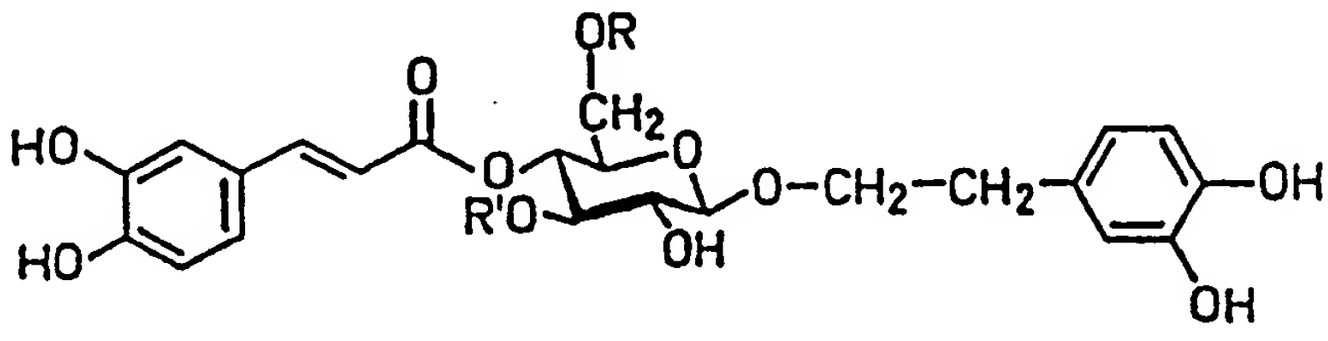
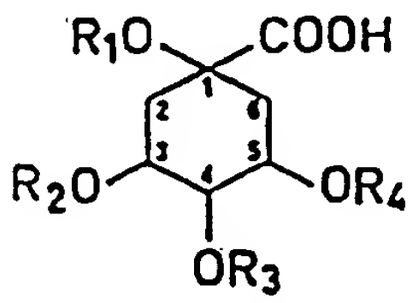
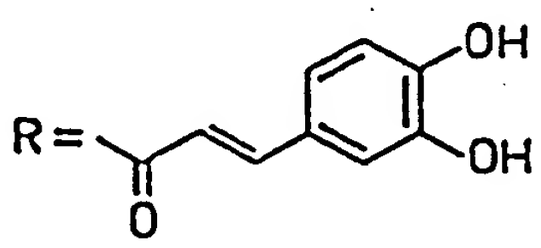

12
Undeca-2E-en-8,10-diinsäure-isobutylamid
(Martin (1965): Acmeta ciliata)
8
Dodeca-2E,4E,8Z,10E-tetraensäure-isobutylamid
13
Undeca-2Z-en-8,10-diinsäure-isobutylamid
9
Dodeca-2E,4E,8Z,10Z-tetraensäure-isobutylamid
14
Dodeca-2E-en-8,10-diinsäure-isobutylamid
18
Pentadeca-2E,9Z-dien-12,14-diinsäure-isobutylamid
(Bohlmann (1963): Echinacea purpurea)
1
Undeca-2E,4Z-dien-8,10-diinsäure-isobutylamid
19
Hexadeca-2E,9Z-dien-12,14-diinsäure-isobutylamid
2
Undeca-2Z,4E-dien-8,10-diinsäure-isobutylamid
(Bohlmann (1966): Echinacea angustifolia/E. purpurea)
11
Dodeca-2E,4E-diensäure-isobutylamid
(Veréb (1978): Echinacea purpurea)
15
Dodeca-2E,4Z,10Z-trien-8-insäure-isobutylamid
6
Trideca-2E,7Z-dien-10,12-diinsäure-isobutylamid
(Bohlmann (1963): Echinacea purpurea)
16
Undeca-2Z-en-8,10-diinsäure-2-methylbutylamid
17
Dodeca-2E-en-8,10-diinsäure-2-methylbutylamid
12
13
14
8,9
18
15
17
16
1
2
6
19
11
0
10
20
(min)

FIG 4



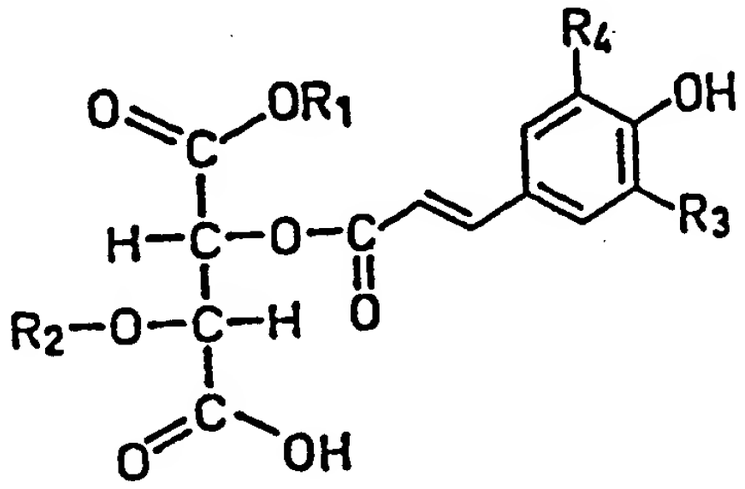
	R	R'
Echinacosid	Glucose (1,6-)	Rhamnose (1,3-)
6-O-Caffeoyl-echinacosid	6-O-Caffeoyl-glucose (1,6-)	Rhamnose (1,3-)
Verbascosid	H	Rhamnose (1,3-)
Desrhamnosyl-verbascosid	H	H

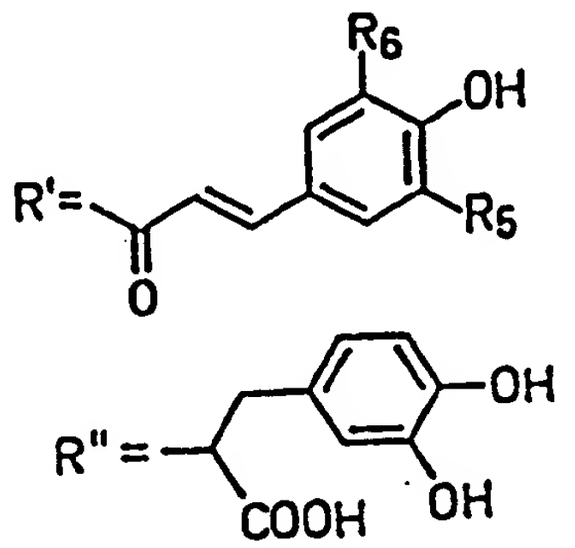




	R_1	R_2	R_3	R_4
3-O-Caffeoyl-chinasäure (Chlorogensäure)	H	R	H	H
Isochlorogensäuren	H	R	R	H
	H	R	H	R
	H	H	R	R
Cynarin	R	H	H	R

FIG 5





	R_1	R_2	R_3	R_4	R_5	R_6
2-O-Caffeoyl-weinsäure (Caftarsäure)	H	H	OH	H	-	-
2,3-O-Di-caffeoyl-weinsäure (Cichoriensäure)	H	R'	OH	H	OH	H
2,3-O-Di-caffeoyl-weinsäure-methylester	CH_3	R'	OH	H	OH	H
2-O-Feruloyl-weinsäure	H	H	OCH_3	H	-	-
2-O-Caffeoyl-3-O-cumaroyl-weinsäure	H	R'	H	H	H	H
2-O-Caffeoyl-3-O-feruloyl-weinsäure	H	R'	OH	H	OCH_3	H
2,3-O-Di-5-[α-carboxy-ß-(3,4-dihydroxy-phenyl)-ethyl]-caffeoyl]-weinsäure	H	R'	OH	R"	OH	R"
2-O-Caffeoyl-3-O-[5-[α-carboxy-ß-(3,4-dihydroxy-phenyl)-ethyl]-caffeoyl]-weinsäure	H	R'	OH	H	OH	R"

FIG. 6

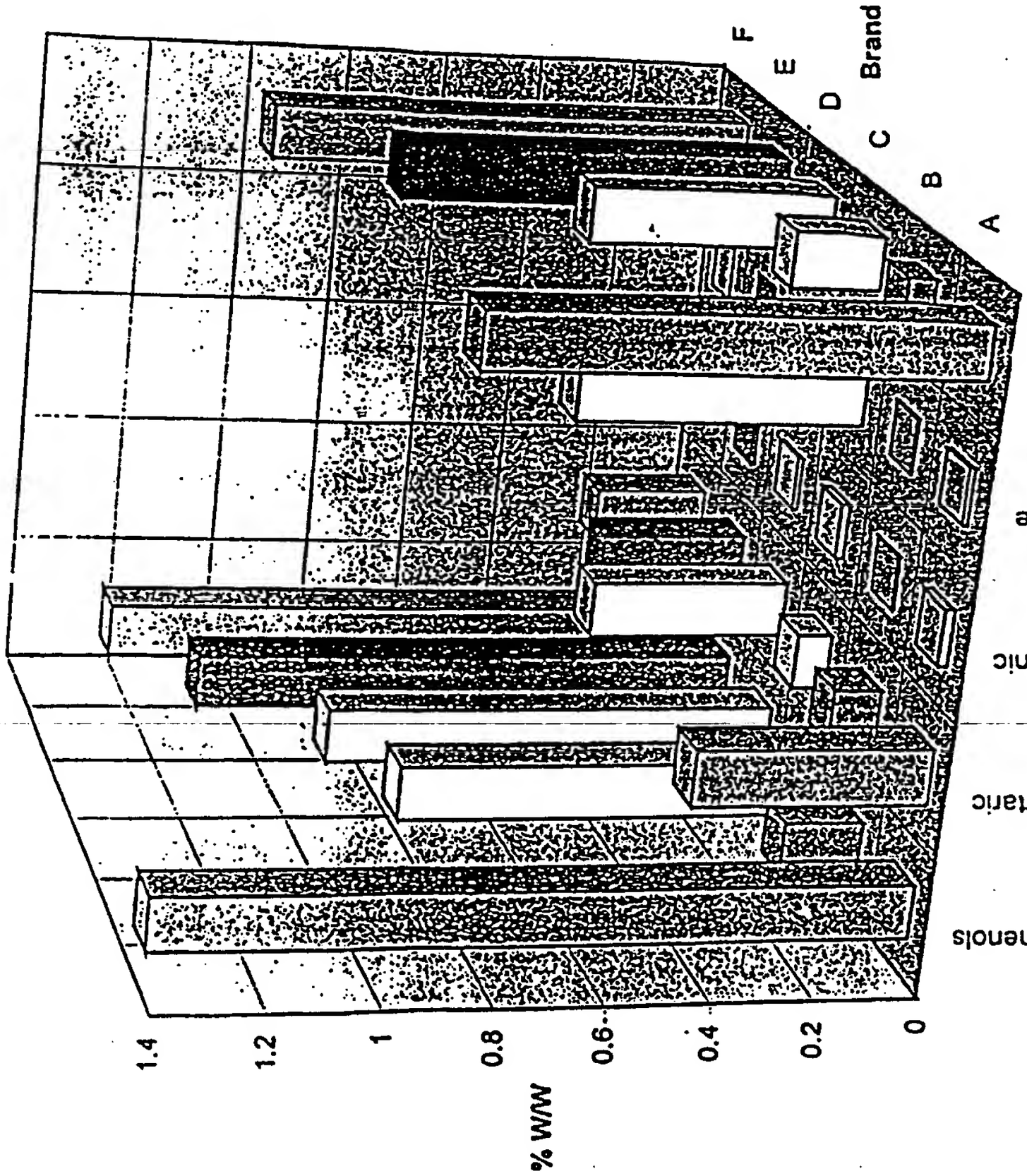

F
E
D
C
Brand
B
A
Cichoric
Echinoside
Chlorogenic
Caffeoyltartaric
Total Phenols
Compound
1.4
1.2
1
0.8
0.6
0.4
0.2
0
% W/W

FIG 4

INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/US 98/22507

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 A61K35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 97 39355 A (PHARMAPRINT INC. & UNIVERSITY OF SOUTHERN CALIFORNIA) 23 October 1997 see claims 1,11 ---	1-27
Y	WO 96 32122 A (UNIVERSITY OF SOUTHERN CALIFORNIA) 17 October 1996 see page 1, line 4 - line 12; claim 1 ---	1-27
Y	EP 0 464 298 A (INDENA SPA) 8 January 1992 see claim 7	1-27
X	-----	27

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 1999	07/04/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Van Bohemen, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte Jonal Application No
PCT/US 98/22507

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